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Molekulární mechanismy nádorové patogeneze signální cesty Hedgehog u vybraných nádorových typů

Molecular mechanisms of tumor pathogenesis of Hedgehog signaling pathway in selected tumor types

Disertační práce

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List of abbreviations

AKT	Protein kinase B
ATO	Arsenic trioxide
AXL	AXL receptor tyrosine kinase
BCC	Basal cell carcinoma
BCL2	B-cell lymphoma 2
BOC	Brother of CDO
BRAF	B-RAF kinase
CDO	CAM-related/downregulated by oncogene
DHH	Desert Hedgehog
DISP	Dispatched
DOX	Doxycycline
DR	Death receptor
E2F1	E2F transcription factor1
EGR1	Early growth response protein 1
EMA	European Medicines Agency
EMT	Epithelial-to-mesenchymal transition
FDA	Food and Drug Administration
GAS1	Growth arrest-specific 1
GLI	Glioma-associated oncogene
GLIA	Activator form of GLI factors
GLIR	Repressor form of GLI factors
GPCR	G protein-coupled receptor
GSK3β	Glycogen synthase kinase 3β
HH	Hedgehog
HHIP	Hedgehog interacting protein
IAP	Inhibitor of apoptosis
iASPP	Apoptosis-stimulating protein of p53
IFT	Intraflagellar transport protein
IFT80	Intraflagellar transport protein 80
IHH	Indian Hedgehog
KLF5	Kruppel-like factor 5
MAPK	Mitogen-activated protein kinase

MD	Medulloblastoma
MEK	MAPKK, Mitogen-activated protein kinase kinase
MITF	Microphthalmia-associated transcription factor
MSH	Melanocyte stimulating hormone
mTOR	Mammalian target of rapamycin
NBCC	Nevoid basal cell carcinoma syndrome
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PC	Primary cilium (cilia)
PKA	Protein kinase A
PTCH1	Patched1
PTCH2	Patched2
S6K1	Ribosomal protein S6 kinase beta-1
SCUBE2	Signal Peptide, CUB And EGF-Like Domain-Containing Protein 2
SFK	SRC family kinases
SHH	Sonic Hedgehog
SMAC/DIABLO	Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein
SMO	Smoothened
SOX2	Sex-determining region Y- BOX2
SP1	Specific protein1
SP3	Specific protein 3
SUFU	Suppressor of Fused
TGF- β	Transforming growth factor – β
TRAIL	Tumor necrosis factor-related ligand
XIAP	X-linked inhibitor of apoptosis protein
ZPA	Zone of polarizing activity

Abstrakt

Předkládaná dizertační práce se zaměřuje na roli signální cesty Hedgehog (HH) v nádorové patogenezi. Signální dráha HH je evolučně velmi konzervovaná signální dráha, která hraje zásadní roli v embryonálním vývoji. V dospělosti je její aktivita silně omezená, aktivovaná je především v kmenových a progenitorových buňkách například mozku, plic, kůže nebo prostaty. Důležitou roli hraje v udržování homeostázy tkání a v jejich regeneraci. Aberantně aktivovaná dráha HH je klíčová v progresi nádorů.

Cílem předkládané práce bylo objasnit nové detaily týkající se signalizace HH dráhy. Podařilo se nám identifikovat nový cílový gen HH dráhy – anti-apoptotický protein survivin, jehož exprese je považována za významný nádorový marker spojovaný se špatnou prognózou pacientů. Prokázali jsme, že inhibitor GANT61, který blokuje koncové proteiny HH dráhy GLI1 a GLI2, snižuje hladinu survivinu v nádorových buňkách. Následně jsme GANT61 spolu s inhibitorem BCL2 proteinové rodiny obatoclaxem použili k inhibici růstu melanomových buněk. Tato kombinace se ukázala velmi efektivní v eradikaci melanomových buněk. Prokázali jsme také, že GANT61 spouští v melanomových buňkách proces apoptózy.

Zjistili jsme také, že signální dráha Hedgehog je aktivovaná u velkého množství buněčných kultur odvozených od různých typů nádorů.

Dále jsme testovali takzvaný reostatový model transkripčního faktoru MITF u melanomu, podle kterého jsou vysoké hladiny MITF spjaté s vysokou diferenciací a malou invazivitou melanomových buněk a nízké hladiny MITF jsou spojené s malou mírou diferenciace, proliferace a vysokou mírou invazivity. Vytvořili jsme buněčný model s inducibilně regulovatelnou hladinou MITF. Pozorovali jsme, že snížení hladiny MITF se neodrazilo na vlastnostech buněk – nesnížila se míra proliferace, ani se nezvýšila invazivita, ale snížila se exprese diferenciačních markerů. To naznačuje, že role transkripčního faktoru MITF musí být dále zkoumána a lépe definována.

Předložené výsledky ukazují na důležitost dráhy HH v nádorové progresi a ukazují na důležitost kombinované cílené terapie.

Klíčová slova: signální dráha Hedgehog, survivin, apoptóza, GANT61, obatoclax, kombinovaná terapie, melanom, MITF

Abstract

The presented doctoral thesis is focused on the role of the Hedgehog (HH) signaling pathway in cancer pathogenesis. HH signaling pathway is an evolutionarily conserved signaling pathway that plays an essential role in embryonic development. Its activity is strictly limited to stem and progenitor cells for example in brain, lung, skin or prostate. HH pathway also plays a key role in tissue homeostasis and regeneration. Aberrantly activated HH pathway is essential in cancer progression.

The aim of the presented thesis was to elucidate new details about the HH signaling pathway. We identified a new target gene of the HH pathway – the anti-apoptotic protein survivin. Survivin is considered to be an important tumor marker associated with a poor prognosis of patients. We showed that the inhibitor of HH pathway effectors GLI1 and GLI2 GANT61 reduced the survivin level in cancer cells. Subsequently, we used GANT61 and the inhibitor of the anti-apoptotic BCL2 protein family obatoclax to inhibit melanoma cells growth. We showed that the combination of these inhibitors was very effective in the eradication of melanoma cells in vitro. We also proved that GANT61 triggers the process of apoptosis in melanoma cells.

We found out that the HH signaling pathway is canonically activated in many cell lines of various tumor origins. Next, we tested the so-called "rheostat model" of MITF transcription factor in melanoma. According to the model, a high-MITF level is associated with high differentiation and low invasion and a low-MITF level is connected with a low differentiation and proliferation rate and high invasion. We established cell lines with inducibly regulated MITF levels. We observed that cell characteristics did not reflect the reduction of MITF level – neither proliferation rate nor invasion decreased. But the expression of differentiation markers decreased. It implies that the role of MITF needs to be more researched and defined better.

The presented results highlight the role of HH signaling in tumor progression and point out the importance of combined therapy.

Key words: Hedgehog signaling pathway, survivin, apoptosis, GANT61, obatoclax, combined therapy, melanoma, MITF

CONTENT

1 INTRODUCTION

Hedgehog (HH) pathway is an evolutionarily conserved signaling cascade. Hedgehog mutation was identified for the first time by Nüsslein-Volhard and Wieschaus in 1980 in large-scale screening for mutations disrupting *Drosophila melanogaster* larval segmentation (Nüsslein-volhard and Wieschaus, 1980). *Drosophila* HH genes were cloned 12 years later and human HH genes were identified and cloned in 1995 (Marigo et al., 1995). In 1993 the sonic hedgehog (SHH) expression was localized to the notochord and the floor plate of the neural tube of developing zebrafish embryo (Krauss et al., 1993). This expression pattern was subsequently shown in chicken. Furthermore, SHH expression was localized to the zone of polarizing activity (ZPA), a region in the posterior of limb buds of a chicken embryo (Echelard et al., 1993; Riddle et al., 1993). Signalization in ZPA is crucial in the formation of the anterior-posterior axis of the developing limb bud. Thus, SHH was established as a key regulator of embryogenesis in vertebrates. An increased interest in the HH pathway followed. Models of HH canonical and non-canonical signaling pathway were created and established. Signaling crosstalk between HH and other signaling pathways has been investigated and the role of HH signaling in embryogenesis and adult tissues has been clarified. In recent years the HH pathway research has been focused on its aberrant activation in various diseases, mainly in cancers. In the presented thesis, I will also focus on this area of HH research.

1.1 HEDGEHOG SIGNALING PATHWAY

1.1.1 CANONICAL HEDGEHOG SIGNALING

Human HH signaling pathway (Fig.1) begins in "HH ligand producing cells" secreting one variant of HH ligand - sonic hedgehog (SHH), Indian Hedgehog (IHH) or desert hedgehog (DHH). The expression of HH ligand is tissue-specific. Before ligand is released from the cell, ligand precursors undergo a maturation process. Precursors are autoproteolytically cleaved and cholesterol is added to C-terminus of newly-emerged N-terminal protein, its N-terminus is palmitoylated. Mature HH ligand is released from the cell via multipass transmembrane protein Dispatched (DISP) acting in synergy with secreted protein SCUBE2 (Signal Peptide, CUB And EGF-Like Domain-Containing Protein 2). Afterward, secreted HH ligand binds to the Patched1 or Patched2 receptor (PTCH1, PTCH2) and its co-receptors on the "receptor cell" membrane. PTCH1, 12-pass transmembrane

receptor, together with three co-receptors CAM-related/downregulated by oncogene (CDO), brother of CDO (BOC) and growth arrest-specific 1 (GAS1) form multimolecular complexes that facilitate transduction of HH signal. (Briscoe and Thérond, 2013)

In the absence of HH ligand, PTCH1 is localized to the base of primary cilia (PC), special non-motile cilia on the surface of epithelial cells. PTCH1 represses and excludes receptor Smoothened (SMO) from the PC. SMO is a 7-pass transmembrane receptor, a member of G protein-coupled receptor (GPCR) superfamily. SMO excluded from PC exists in the form of inactive dimers. Protein Suppressor of Fused (SUFU) blocks effectors of HH pathway - Glioma-associated oncogene (GLI) transcription factors. The SUFU/GLI complex is localized in the tip of PC. Protein kinase A (PKA) and glycogen synthase kinase 3β (GSK3β) promote the formation of repressor forms of GLI factors (GLIR) that are subsequently translocated to the nucleus, where they bind to the HH target gene promoters and block their transcription (Briscoe and Thérond, 2013; Cochrane et al., 2015).

When the HH ligand binds to PTCH1, the repression of SMO is relieved. SMO enters into the primary cilia and represses the activity of SUFU. It results in SUFU/GLI complex dissociation. GLI factors are post-translationally modified and form activator forms (GLIA) which are translocated to the nucleus, where they activate the expression of target genes (Skoda et al., 2018; Wheway et al., 2018).

HH signaling pathway has three effectors GLI1, GLI2 and GLI3. They are zinc-finger transcription factors. GLI1 contains C-terminal activator domain and is considered an activator of the pathway. It has a role in amplifying transcriptional response via induction of expression of the *GLI1* gene and making positive feedback of HH signaling (Park et al., 2000; Regl et al., 2002). GLI2 and GLI3 consist of N-terminal repressor domain and C-terminal activator domain and act dependently on cellular context. GLI2 is considered an activator of the pathway and GLI3 is mainly thought to be a repressor (Skoda et al., 2018).

HH pathway activates many target genes. HH signaling regulates the expression of genes coding the components of HH pathway such as *PTCH1, PTCH2, HHIP* (Hedgehog Interacting protein, the negative regulator of HH signaling) and *GLI1*. Other target genes are essential in different cellular processes as cell cycle (e.g. *CCND1, CCND2, FOXM1*), regulation of apoptosis (*BCL2, CFLAR*), epithelial-to-mesenchymal transition (*FOXC2, SNAI1, TWIST2, ZEB1, ZEB2*), stem-cells signaling (e.g. *WNT2B, WNT5A, JAG2*). HH pathway also regulates the expression of stem cell markers (*CD44, CD133, LGR5*) (Katoh and Katoh, 2009).
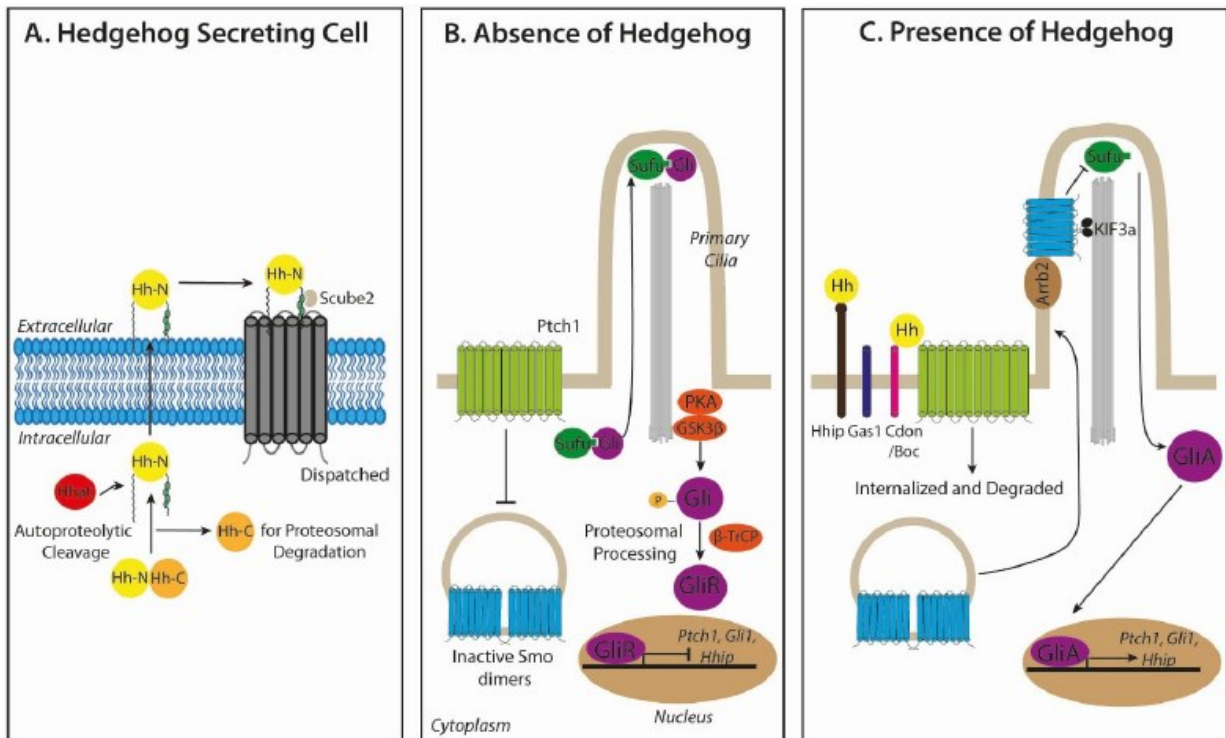
Fig.1 Canonical Hedgehog signaling pathway. A/*Precursor of HH ligand is autoproteolytically cleaved and newly-emerged N-terminal protein is palmitoylated on its N-terminus and cholesterol is added to its C-terminus. The HH protein is released via Dispatched acting in synergy with SCUBE2. **B/** In the absence of HH ligand PTCH1 is localized to the base of primary cilia and inhibits SMO receptor. The SUFU/GLI complex is localized in the primary cilia and recruiting of kinases PKA and GSK3β and β-TRCP complex leads to the formation of GLI repressor forms that are translocated to the nucleus and block transcription of HH target genes. **C/** In the presence of HH ligand, PTCH1 releases SMO. SMO enters the primary cilia and represses SUFU. It leads to the releasing of activator forms of GLI factors. GLIAs are translocated to the cell nucleus and activate the expression of HH target genes. (Cochrane et al., 2015, licensed under Creative Commons Attribution license (http://creativecommons.org/licenses/by/4.0/))*

1.1.2 NON-CANONICAL HEDGEHOG SIGNALING

HH signaling pathway can also be activated non-canonically, which means that HH signaling differs from the standard HH-PTCH-SMO-GLI axis. There is confusion in literature and scientific papers about what does it mean.

The first type of non-canonical signaling is often called signaling crosstalk. It means that GLI factors are activated by proteins from other signaling pathways and the HH pathway upstream of GLI factors is excluded from signalization. The importance of this type of signaling is highlighted by the number of signaling pathways activating GLI factors. Most of these pathways are essential in embryonic development and important cellular processes. For example, the mTOR (mammalian target of rapamycin) signaling pathway affects GLI factors in many ways. PI3K-AKT-mTOR signaling pathway increases GLI2 transcriptional activity via AKT (protein kinase B), preventing proteasomal degradation of GLI2 (Riobó et al., 2006).

Interestingly, canonical HH ligand signalization induces the expression of DYRK1B kinase leading to activation of the mTOR/AKT pathway. It promotes AKT-mediated stability of GLI proteins (Singh et al., 2017). In esophageal carcinoma mTOR/S6K1 (ribosomal protein S6 kinase beta-1) signaling pathway promotes GLI1 activity by its phosphorylation leading to the release of GLI1 from the SUFU complex (Wang et al., 2012).

TGF-β (transforming growth factor – β) is another factor non-canonically activating GLI proteins. TGF-β induces expression of GLI1 and GLI2. TGF-β directly activates GLI2, but GLI1 expression is activated in a GLI2 dependent manner (Dennler et al., 2009; 2007). Interestingly, the signaling pathway RAS-RAF-MEK-ERK enhances GLI transcriptional activity (Gu and Xie, 2015), whereas MAPKKK/MEKK axis inhibits the transcriptional activity of GLI (Pietrobono et al., 2019). Many other signaling pathways or molecules activate GLI transcription factors independently of SMO (Pietrobono et al., 2019), highlighting the role of signaling crosstalk.

The second type of non-canonical signaling occurs in a GLI-independent manner. SMO does not activate GLI factors. Instead, SMO coupled with G-protein of the Gαi family modulates Ca^{2+} flux, activation of the small GTPases RHOA and RAC1 and Warburg-like metabolism (Arensdorf et al., 2016). It was shown that the canonical HH pathway is not active in human endothelial cells, but HH ligand-SMO-Gαi-RAC1-RHOA signaling stimulates the formation of actin stress fibers and endothelial cell tubulogenesis (Chinchilla et al., 2010). The role of intraflagellar transport proteins (IFT) in HH signal transduction is interesting, too. IFT80 promotes canonical HH signaling in murine osteoblasts precursor cells. It was shown that loss of IFT80 blocks canonical signaling and promotes non-canonical HH–Gαi–RHOA–stress fiber signaling (Yuan et al., 2016). That study reveals the importance of IFT80 in balancing between non-canonical and canonical HH signaling pathways, minimally in murine osteoblasts, and indicates how HH signaling pathways can be switched in cells.

Importantly, non-canonical HH signaling plays a role in axon guidance. It was described that axons of commissural neurons react on increasing concentrations of SHH. SHH activates SRC family kinases (SFK) in a SMO-dependent manner and activated SFK mediates changes in axon growth cone cytoskeleton in reaction to SHH gradient (Yam et al., 2009).

HH non-canonical signaling can also occur in a SMO-independent manner and lead to increased cell proliferation and survival. It was proved that PTCH1 in the absence of SHH induces apoptosis. Pro-apoptotic signalization of PTCH1 is disrupted in the presence of SHH.

Thus, PTCH1 is considered a dependence receptor acting differently in SHH ligand's presence or absence (Mille et al., 2009).

1.2 HEDGEHOG SIGNALING IN DEVELOPMENT

As mentioned above, HH signaling plays a crucial role in the development of all vertebrates. Although all three HH ligands can bind to the PTCH1 receptor, they have different cell and tissue expression patterns. SHH is the most broadly expressed HH ligand. SHH expression appears in early embryogenesis and has an essential role in the central nervous system. SHH is detected in the notochord and the floor plate of neural tube and in the limb bud (Echelard et al., 1993). SHH plays a role in foregut, lung, teeth, muscle, prostate, pancreas and hair follicle development (Ingham and McMahon, 2001). IHH and DHH are localized to a limited number of tissues. IHH is essential for skeletal and bone development (Ohba, 2020; St-Jacques et al., 1999). Furthermore, IHH has a role in hematopoiesis (Dyer et al., 2001) and gastrointestinal tract development (Ramalho-Santos et al., 2000). DHH expression is localized to the male gonads, including Sertoli cells and has a crucial role in the regulation of mammalian spermatogenesis (Bitgood et al., 1996). DHH is also expressed in the peripheral nervous system (Parmantier et al., 1999).

HH signaling is tissue-specific in an adult organism. HH expression is localized to the stem and progenitor cells for example in brain, skin or prostate. HH signaling is important for tissue homeostasis and regeneration (Petrova and Joyner, 2014).

1.2.1 ABERRANT HEDGEHOG SIGNALING IN DEVELOPMENT

Aberrant activation of HH pathway in development leads to developmental defects and malformations. Knockout mutations of *Ptch1, Smo* and *Sufu* are embryonic lethal in mice, causing holoprosencephaly or severe defects of the neural tube (Goodrich et al., 1997; Svärd et al., 2006; Zhang et al., 2001). Viable mutations cause a wide range of developmental defects as holoprosencephaly and craniofacial defects, diverse neural tube defects, defects of skeletal development, congenital disorders, abnormalities in lung, heart or eye development and many others (Sasai et al., 2019). Specific mutations of HH pathway components are linked with cancer development (will be discussed separately in the text below).

It is necessary to point out that not only mutations of HH pathway component but also dysfunctions of primary cilia lead to disruption of HH signaling. For example, mutations in

IFT proteins can disturb the trafficking and distribution of HH pathway proteins (e.g. SMO, GLI, SUFU) in primary cilia. Thus, the disruption of HH signaling pathway may be responsible for the origin of specific developmental abnormalities, such as polydactyly or neural tube defects observed in ciliopathies (Reiter and Leroux, 2017).

1.3 HEDGEHOG SIGNALING IN CANCER

1.3.1 ACTIVATION OF HEDGEHOG SIGNALING IN CANCER

In view of the fact that the aberrantly activated HH signaling pathway causes severe developmental defects, it is not surprising that its aberrant somatic activation causes different malignancies. Three different models of HH signaling activation were proposed (Scales and de Sauvage, 2009):

Type I - HH ligand-independent signaling driven by mutations

Type II - HH ligand-dependent signaling in autocrine manner

Type III- HH ligand-dependent signaling in paracrine manner

Type I HH signaling independent of HH ligand is activated via mutated components of the HH pathway. These mutations were found at the very beginning of the HH research. HH signaling was linked with cancer for the first time when a mutation in *PTCH1* was discovered to be a cause of autosomal dominant genetic disorder Gorlin syndrome, also called nevoid basal cell carcinoma (NBCC) syndrome. NBCC patients suffer from multiple basal cell carcinomas (BCC) at a young age. BCC development is linked with loss of heterozygosity in *PTCH1* gene, leading to the constitutive activation of HH signaling pathway. *PTCH1* is considered to be a tumor suppressor gene (Hahn et al., 1996). Similarly, in sporadic (nonhereditary) BCCs *PTCH1* inactivating mutations are found in high frequency (Gailani et al., 1996) and also activating mutations of *SMO* are found in some cases (Xie et al., 1998). The second type of tumor connected to NBCC is medulloblastoma (MD), a malignant brain tumor occurring in children (Schofield et al., 1995). About 5% of patients suffering from NBCC develop medulloblastoma (Evans et al., 1993). In sporadic MD, loss-of-function mutations of *PTCH1* are found (Pietsch et al., 1997). Somatic and germline mutations of *SUFU* are also found in MD patients (Brugières et al., 2012; Taylor et al., 2002).

Type II signaling does not need mutations in components of the HH pathway. Instead, the HH ligand is overexpressed and activates HH signaling in an autocrine manner. It means that the activated HH pathway stimulates the proliferation or survival of producing cell itself. This type of signaling was described in various cancers that originated from epithelial tissues, including prostate (Bushman, 2016), gastrointestinal tract - esophagus, stomach, biliary tract and pancreas (Berman et al., 2003), lung (Szczepny et al., 2017; Watkins et al., 2003), or breast (Hui et al., 2013).

In type III signaling, the activated HH pathway acts in a paracrine manner. This type of signaling is crucial in the development of various tissues in embryogenesis and in the maintenance of stem cells in the adult organism, e.g. in the gastrointestinal tract (Kolterud et al., 2009). Paracrine activation has been reported in prostate, colorectal or esophageal cancers (Sari et al., 2018). Paracrine signaling has been studied in recent years in pancreatic cancer. Tian et al. showed that the oncogenic allele of *Smo* did not cause activation of the HH pathway in epithelial cells but activated HH signaling in mesenchymal cells of tumor stroma in mice. The authors observed that the HH pathway is active in the tumor stroma surrounded by tumor epithelial cells expressing HH ligand (Tian et al., 2009). Another study from 2009 reported similar results indicating HH paracrine signaling between pancreatic epithelial cells and stromal cells. Moreover, the authors did not observe primary cilia that are essential for HH signaling in tumor cells. On the other hand, primary cilia were shown in stromal cells. These facts taken together indicate the decrease of autocrine signaling in tumor cells and paracrine activation in tumor stroma (Bailey et al., 2009). Similar but "reverse" paracrine signaling appears in hematological malignancies, especially in B-cell malignancies. In this case, HH ligand, which is produced in bone marrow cells, stromal cells of the spleen and lymph nodes, has a paracrine effect on neoplastic cells of B-cell malignancies (Dierks et al., 2007). This "reverse" paracrine signaling is sometimes called type IV of HH signaling.

1.3.2 HEDGEHOG SIGNALING IN TUMOR PROGRESSION

Increased expression of SHH and activated HH signaling pathway play an important role in cancer progression. Many cellular and tissue processes are deregulated in tumor cells. Many of them are affected or deregulated by the activated HH pathway (Fig. 2). For example, HH signaling plays an essential role in proliferation, escape from apoptosis, reactivation of telomerase activity, angiogenesis, deregulation of energetic metabolism, epithelial-to-mesenchymal transition, escaping the immune system, activation of invasion and metastasis

or genomic instability (Hanna and Shevde, 2016). Here will be described just those processes that are relevant to the thesis.

Fig. 2 Role of HH signaling in tumor initiation, progression and metastasis. *In the picture, the main processes affected by HH signaling in tumors are highlighted. (Hanna and Shevde, 2016, licensed under Creative Commons Attribution International License (http://creativecommons.org/licenses/by/4.0/))*

1.3.2.1 Hedgehog signaling in apoptosis

Apoptosis is a cellular process of programmed cell death. Apoptosis is often pathologically blocked in cancers. It allows cancer cells to continue in growth. Defects in apoptotic pathways lead to malignant transformation of affected cells, metastasis of tumor cells and resistance to anticancer drugs (Wong, 2011). The connection between the HH pathway and apoptosis in development and cancer has been shown in various studies. As mentioned above, PTCH1 receptor in the absence of HH ligand induces apoptosis. The binding of HH ligand activates the HH pathway downstream of PTCH1 and blocks the pro-apoptotic activity of PTCH1 (Mille et al., 2009). Conversely, it was shown that blocking of

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SHH has an antitumor effect and restores PTCH1-induced apoptosis in colon, lung and pancreatic cancer cells (Bissey et al., 2020).

Apoptosis can be induced in cancer cells by treatment with HH pathway inhibitors. For example, Ma and colleagues observed increased apoptosis in esophageal cancer cells after treatment with SMO inhibitors (Ma et al., 2006). Similar results were shown in a study focusing on cerebellar progenitor cells and also on medulloblastoma cancer cells. Apoptosis was induced by SMO antagonists (Noguchi et al., 2015).

The connection between the HH pathway and apoptosis was also proved on a molecular basis. HH pathway affects the expression of pro-apoptotic protein Noxa. It was shown that GLI1 overexpression decreased the expression of Noxa in *TP53*-mutated rhabdomyosarcoma and medulloblastoma cells. Treatment of cancer cells with GANT61 (inhibitor of GLIs) resulted in increased expression of Noxa. The results of the study indicate that GLI1 blocks Noxa protein indirectly and the authors suggest that GLI1 represses EGR1 (early growth response protein 1) expression, which subsequently represses the transcription of Noxa (Meister et al., 2018).

HH signaling also affects the expression of anti-apoptotic proteins. Two research groups identified the anti-apoptotic gene *BCL2* (B-cell lymphoma 2) as a direct target gene of the HH pathway, although the researchers disagreed on the exact mechanism of *BCL2* activation. Regl and colleagues identified GLI2 protein as a factor activating *BCL2* expression (Regl et al., 2004), whereas Bigelow et al. determined GLI1 as the main activator of *BCL2* (Bigelow et al., 2004). Another anti-apoptotic protein XIAP (X-linked inhibitor of apoptosis protein) is also directly regulated by GLI2. *XIAP* gene was determined as direct transcriptional target of GLI2 (Kurita et al., 2011).

Moreover, Kurita and colleagues linked HH signaling and apoptosis differently. They studied the extrinsic apoptotic pathway, initiated by the apoptotic ligand (FAS, TWEAK or TRAIL) binding to the membrane receptor transducing apoptotic signals into the cell. TRAIL (Tumor necrosis factor-related ligand) ligand binds to receptors DR4 or DR5 (death receptor 4 or 5, respectively). Kurita's group showed that GLI3 (but not GLI1 or GLI2) blocks DR4 expression and thus blocks TRAIL-ligand-based apoptosis in cholangiocarcinoma cells (Kurita et al., 2010). Moreover, the HH pathway can modulate the switch between intrinsic and extrinsic (TRAIL-based) apoptotic pathways. As mentioned above, the anti-apoptotic gene *XIAP* is a target gene of GLI2. XIAP represses the extrinsic apoptotic pathways, but blocking HH signaling (and XIAP) by anticancerous drug leads to activation of extrinsic mitochondria- independent pathway (Kurita et al., 2011). Taken together, it seems that the

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activated HH pathway acts as a suppressor of apoptosis in cancer cells, but more research is needed.

1.3.2.2 Hedgehog signaling in epithelial-to-mesenchymal transition

The epithelial-to-mesenchymal transition (EMT) is a cellular process essential in embryonic development, tissue regeneration, organ fibrosis and wound healing. The epithelial phenotype of cells changes into mesenchymal phenotype in EMT. EMT is important in tumor progression and allows metastatic progression. The switch among epithelial and mesenchymal state is triggered by activating of transcription factors ZEB1, ZEB2, SNAI1, SNAI2 or TWIST (Roche, 2018), most of them is upregulated in HH signaling dependent manner (ZEB1, ZEB2, SNAI1, TWIST2) (Katoh and Katoh, 2009). Loss of E-cadherin is also typical for EMT, other EMT markers as vimentin, fibronectin and N-cadherin are elevated (Gonzalez and Medici, 2014). HH signaling has been linked with EMT in various cancers, e.g. in breast cancer vimentin and SNAIl were elevated in SHH/GLI1 positive patients. HH pathway blocking leads to lowered cell viability, induced apoptosis and altered expression levels of EMT markers (Riaz et al., 2019). Similarly, the role of HH signaling in EMT was shown in muscle-invasive bladder cancer progression (Kitagawa et al., 2019) or esophageal adenocarcinoma (Wang et al., 2018). EMT activation is also a crucial regulator of cancer stem cell (CSC) phenotype (Shibue and Weinberg, 2017). In pancreatic cancer stem-like cells, it was shown that blocking of HH pathway resulted in inhibition of EMT, self-renewal of CSCs, invasion, chemoresistance, or pulmonary metastasis (Wang et al., 2016).

1.3.2.3 Hedgehog signaling in cancer stem cells

CSCs are tumor cells displaying the capability of self-renewal and differentiation into specialized cell types. CSCs are resistant to radiation treatment and chemotherapy and therefore play a crucial role in cancer relapse. They are believed to be the origin of cancer metastasis (Yu et al., 2012). Signaling pathways important in embryonic development as Notch, HH or WNT are aberrantly activated in CSCs (Takebe et al., 2015). As mentioned above HH pathway plays a role in the process of EMT in CSCs, e.g. in pancreatic CSCs (Wang et al, 2016). HH pathway contributes to the maintenance of CSCs stemness by regulation of expression of important stemness genes such as *NANOG, OCT4, SOX2* and *BMI* (Clement et al., 2007; Po et al., 2010). The essential role of HH signaling in CSCs maintenance was shown in chronic myeloid leukemia (Zhao et al., 2009) and other

hematological cancer (Campbell and Copland, 2015) and also in solid tumors, such as lung cancers, breast and prostate cancer or glioma (Cochrane et al., 2015).

1.4 HEDGEHOG SIGNALING IN MALIGNANT MELANOMA

1.4.1 MALIGNANT MELANOMA

Malignant melanoma is the most aggressive type of skin cancer. Initial stages are cured by surgery and the 5-year survival rate is 90-100%, but highly aggressive metastatic malignant melanoma patients have a median survival rate from six to ten months (Bertolotto, 2013). The incidence of newly diagnosed cases increases rapidly. The risk of developing melanoma is associated with skin exposure to UV light, especially to the UV-B part of the spectrum.

Melanoma originates from the melanocytes, neural crest-derived cells. Melanocytes are localized mainly in the basal epidermis and hair follicle. UV-light causes DNA damage in skin keratinocytes, which in response produce MSH (melanocyte stimulating hormone). Subsequently, MSH binds the GPCR receptor melanocortin receptor 1 of melanocytes and activates the production of melanin in melanosomes, the lysosome-like structures. Melanosomes carrying melanin are trafficked to the keratinocytes, where they are placed over the "sun-exposed" side of nuclei and protect the nuclei from the UV-light (Lin and Fisher, 2007). Melanocytes can escape from the control of keratinocytes, proliferate and spread and form a naevus or common mole. Melanoma derives from preexisting nevi in 25 % and transformed melanocytes in 75 % cases (Berlotto, 2013).

Mutations in various genes in melanoma precursor cells or lesions are responsible for tumor initiation and progression. Mutations in the MAKP/ERK signaling pathway are crucial for melanoma development. *NRAS* gene is mutated in about 15 % of melanomas. The most common NRAS mutation occurs at position 61 of the protein (80% from all NRAS mutations) and less frequent are mutations in positions 12 and 13 (Savoia et al., 2019). Another frequently mutated gene is *BRAF*, which is mutated in approximately 60 % of melanoma cases. The most frequent mutation is the V600E mutation, occurring in 80-90% of all BRAF (B-RAF kinase) mutations. V600E mutation is a result of the substitution of amino acid valine (V) to glutamic acid (E) at position 600 of the protein chain (Davies et al., 2002). Interestingly, V600E mutation is observed in 80 % of benign nevi conferring the oncogene-induced senescence on these cells (Michaloglou et al., 2005). This finding indicates that

BRAF(V600E) is critical in the initiation of melanoma development but alone is not sufficient for melanoma tumorigenesis (Michaloglou et al., 2005; Pollock et al., 2003). Mutations of other proteins from the MAPK/ERK pathway are also observed – mutations in MEK are reported in 6-7 % of melanoma cases, ERK in less than 1%. (Savoia et al., 2019).

PI3K-AKT signaling pathway is also often mutated in melanomas. It is activated in 10-30 % of melanomas (Davies, 2012). Other signaling pathways important for melanoma tumorigenesis are WNT, JNK/c-JUN, JNK/ATF2, NF-κB or JAK/STAT (Lopez-Bergami et al., 2008).

1.4.2 MITF IN MELANOMA

Melanoma research is focused on the MITF transcription factor (microphthalmia-associated transcription factor). MITF is basic-helix-loop-helix leucine zipper transcription factor. MITF has several tissue-specific isoforms that differ in the first exon and each isoform has a specific promoter. Isoform M-MITF is melanocyte and melanoma-specific (written as MITF in the text below) (Wellbrock and Arozarena, 2015). MITF is essential in melanocyte development and is also a key factor in melanoma development. Therefore, MITF can be termed as lineage survival oncogene (Garraway et al., 2005). MITF expression is positively regulated mainly by four transcription factors CREB, SOX10, LEF1 and PAX3 (Bertolotto et al., 1998; Bondurand et al., 2000; Kubic et al., 2008; Takeda et al., 2000). Oppositely, BRN2 can repress MITF expression (Goodall et al., 2008). MITF regulates the expression of many target genes. They can be separated into few groups (reviewed in Cheli et al., 2010):

- Genes important for melanocytes differentiation and melanin synthesis (*TYR, TYRP1, DCT, MLANA, SILV, OA1*)
- Genes controlling cell cycle and proliferation (*TBX2, CDK2, p21, p16*)
- Genes playing a role in cell survival and apoptosis (*BCL2, BIRC7, MET, APEX1/Ref1*)
- Genes playing a role in EMT (*SNAI2*)
- Other genes (*GPNMB/osteoactivin, TRPM1, BEST1/VMD2*)

MITF expression levels were linked with tumor characteristics, such as tumor growth, survival and proliferation of tumor cells, invasion and metastasis. Therefore, the so-called "MITF rheostat model" was created (Carreira et al., 2006; Hoek and Goding, 2010). According to the model, there are subpopulations of tumor cells with distinct MITF levels and with distinct characteristics in the tumor (Fig.3). Tumor cells with high expression of MITF

are associated with differentiation and increased proliferation but low invasion. Oppositely, tumor cells expressing low levels of MITF are more invasive and their proliferation is decreased. Cells with depleted MITF (for example by RNAi) are associated with cell senescence and increased apoptosis.
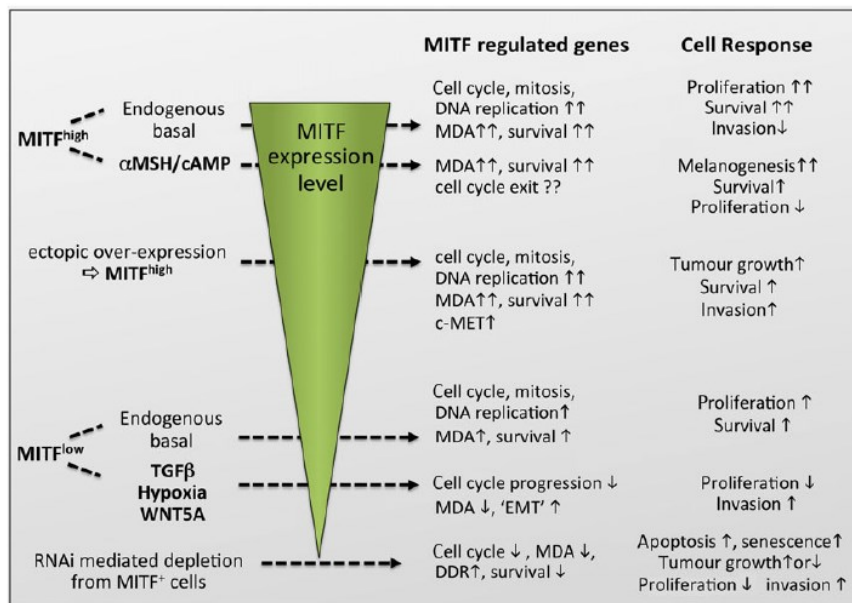
Fig. 3 MITF expression levels linked with expression of MITF regulated genes and cellular responses (model). *(Wellbrock and Arozarena, 2015, licensed under Creative Commons Attribution International License)*

1.4.3 HEDGEHOG SIGNALING IN MELANOMA

Even though HH signaling is not in the focus of melanoma research, some interesting findings were published. It was shown that HH signaling affects processes linked to invasion, proliferation and tumorigenesis of melanoma cells.

Stecca and colleagues showed that RAS-MEK/AKT signaling pathway regulates GLI1 activity in melanoma, highlighting the role of HH-GLI1 signaling in RAS-induced melanomas (Stecca et al., 2007). It was also proved that HH signaling modulates the expression of E2F1 (E2F transcription factor 1) protein that is crucial in cell cycle progression, DNA damage response and apoptosis and is aberrantly activated in various cancers. Moreover, it was shown that HH/GLI-E2F1 axis positively modulates the inhibitor of apoptosis-stimulating protein of p53 (iASPP) in melanoma (Pandolfi et al., 2015).

Interestingly, there is a connection between TGF-β and GLI2 in melanoma. As mentioned above, GLI2 is a direct target of TGF-β (Dennler et al., 2007 and 2009). TGF-β

was shown to play an essential role in melanoma tumorigenicity and invasion (Javelaud et al., 2007; 2005). Alexaki et al. (2010) showed that GLI2 has an important role in invasion and metastasis in melanoma. Increased expression of GLI2 elevated invasion and migration of melanoma cells and also increased the number of bone metastasis in mice and vice versa decreased expression of GLI2 inhibited basal and TGF-β induced cell migration and invasion and decreased number of metastasis.

It was also reported that GLI2 blocks the expression of melanoma transcription factor MITF. It was shown that GLI2 and MITF are inversely correlated. Experiments imply that GLI2 represses expression of MITF and vice versa, independently of BRAF-activating mutations (Javelaud et al., 2011). Subsequently, it was shown that MITF expression is blocked not only by GLI2 but also by TGF-β (Pierrat et al., 2012).

The role of GLI1 in melanoma invasion and metastasis was also shown. Knockdown of *Gli1* blocked invasion and migration of melanoma cells independently of MITF expression level and decreased lung metastasis in mice. Moreover, knockdown of *Gli1* caused reversal process to EMT and melanoma cells gained characteristics of epithelial cells including an increase of E-cadherin and a decrease of N-cadherin and EMT factors as SNAI1, ZEB1, TWIST1 (Gunarta et al., 2017).

Interestingly, HH signaling has recently been connected to intercellular communication of cancer cells affecting e.g. EMT or proliferation of target cells. The significant role of IHH signaling in intercellular communication was shown in melanoma (Arasu et al., 2020).

1.5 TARGETING THE HEDGEHOG PATHWAY

Targeted therapy is a type of drug treatment that specifically blocks target genes involved in cancer cells' growth and survival. Specific inhibitors can block the HH pathway at different levels depending on the protein of HH pathway that is inhibited. Most of the inhibitors target the SMO receptor. The first promising agent inhibiting SMO was cyclopamine, a steroidal alkaloid isolated from the plant *Veratrum californicum* (Chen et al., 2002). The use of cyclopamine in clinical practice has been limited due to low water solubility and low stability in acids and not optional pharmacokinetics. These problems led to the synthesis of more stable derivates of cyclopamine, such as vismodegib (GDC-0449), sonidegib (erismodegib, LDE-225) or saridegib (patidegib, IPI-926). In 2012, vismodegib was approved by the United States Food and Drug Administration (FDA) as standard therapy in

patients with recurrent, locally advanced or metastatic BCC (Axelson et al., 2013). Subsequently, vismodegib was approved by European Medicines Agency (EMA) in 2013. Vismodegib has been tested in many BCC clinical trials and has been tested as a treatment of various cancers (different from BCC). For example, vismodegib showed activity in SHH-driven medulloblastoma (Robinson et al., 2015). On the other hand, no signal of clinical activity (e.g. tumor size reduction) was shown in patients with metastatic castration-resistant prostate cancer, albeit *GLI1* mRNA expression was suppressed (Maughan et al., 2016).

Sonidegib, another inhibitor of SMO, was approved by FDA and EMA in 2015. It was approved as a treatment of locally advanced BCC not suitable for surgery or radiotherapy (Casey et al., 2017). In 2018, the FDA approved SMO inhibitor glasdegib to be used with low doses of cytarabine for the treatment of acute myeloid leukemia in patients older than 75 years or with comorbidities not enabling intensive induction chemotherapy (Norsworthy et al., 2019). Moreover, SMO inhibitors sonidegib, glasdegib, patidegib and itraconazole are in phase III of clinical trials (www.clinicaltrials.gov). Itraconazole is a commonly used antifungal and is also considered a SMO inhibitor, although it does not directly block SMO. Itraconazole blocks the HH pathway by preventing SMO accumulation in primary cilia (Kim et al., 2010).

The problem of SMO inhibitors is acquired resistance. SMO is often mutated in tumors or develops adaptive mutations after the treatment with SMO inhibitors (Pricl et al., 2015). Therefore, inhibitors targeting downstream of SMO, especially drugs blocking GLI factors, are in the focus of the research. For example, genistein, an isoflavone from soybeans, has potent anticancer activity and it was proven that it blocks GLI1, although the exact mechanism of GLI1 blocking is not fully understood (Li et al., 2019a; Zhang et al., 2012). Another GLI inhibitor is arsenic trioxide (ATO), a chemical compound approved by the FDA and used to treat acute promyelocytic leukemia (Beauchamp et al., 2011). GANT58 and GANT61 are direct inhibitors of GLI1 and GLI2 transcription factors. GANT61 is intensively investigated in preclinical studies of various cancer types as rhabdomyosarcoma, neuroblastoma, colon, pancreas, leukemia, prostate or melanoma (Gonnissen et al., 2015). GANT61 has not yet been included in any clinical trial.

SMO-acquired resistance is also possible to solve by combination therapy using HH pathway inhibitors together with inhibitors of other signaling pathways, ionizing radiation or chemotherapy. For example, the combination of HH pathway inhibitors (vismodegib or ATO) with FDA-approved anticancer agents (cisplatin, ifosfamide and doxorubicin) lead to synergistically prevented growth of osteosarcoma in vitro and in vivo (Saitoh et al., 2016).

Combination treatment strategies, including HH pathway inhibition combined with standard cancer treatment, are in the focus of clinical trials. Many clinical trials in phase I or II have already been finished and many are still ongoing (Xie et al., 2019; www.clinicaltrials.gov).

HH pathway inhibition can also be helpful in tumors resistant to radiotherapy or standard therapy. In those tumors, the inhibition of HH pathway can lead to sensitizing to the therapy. For example, GANT61 treatment sensitized prostate cancer cells to radiation in vitro and in vivo (Gonnissen et al., 2016). It was shown that melanoma cell lines with acquired vemurafenib resistance showed increased levels of non-canonically activated GLI1 and GLI2. Treatment with GANT61 restored the sensitivity to vemurafenib in treated cells suggesting that HH pathway inhibition could be an effective strategy in vemurafenib-resistant melanomas (Faião-Flores et al., 2017).

2 AIMS

This study is focused on the role of the Hedgehog signaling pathway in cancers. The study aimed at clarifying new aspects of HH signaling in cancer.

Major aims:

1/ HH pathway is aberrantly activated in many types of cancer. The effectors of the HH pathway, GLI transcription factors, activate a wide range of target genes in tumors. Anti-apoptotic proteins BCL2 and XIAP are direct targets of the HH pathway. Thus, it seems HH signaling has a certain role in disbalancing pro-apoptotic and anti-apoptotic signals in tumor cells. **Our aim was to identify new pro-/anti-apoptotic target gene(s) of the HH pathway and shed more light on the role of HH signaling in apoptosis in cancer cells.**

2/ In recent decades, there are efforts to treat patients suffering from various cancers with a therapy specifically targeted to the tumor. The FDA approved three SMO inhibitors to be used in patients suffering from basal cell carcinoma and acute myeloid leukemia. However, there is a problem with SMO acquired resistance. Therefore, inhibitors of other components of the HH pathway are intensively studied. The research is also focused on therapy combining two or more inhibitors that target different signaling pathways. W**e aimed to examine the HH signaling inhibition by the inhibitor of GLI transcription factors GANT61.**

3/ Our next aim was to find an appropriate combination of inhibitor GANT61 and inhibitor of another signaling pathway that will eliminate cancer cells more effectively than the monotherapy of each agent.

4/ We were also interested in the effect of GANT61 on cancer cells. We aimed to study which tumor characteristics and cellular processes are altered by GANT61 treatment.

5/ There are several different mechanisms of activation of HH signaling pathway, for example HH ligand can be aberrantly overproduced, gain-of-function or loss-of-function mutations of HH pathway components are found. GLI factors can be activated non-canonically. Thus, the expression pattern of the HH pathway components could indicates whether the HH pathway is active (and how is activated) in cancer cells. **Our aim was to examine the completeness of expression of HH pathway components in the cell lines of various tumor origins**.

Minor aims:

5/ MITF transcription factor is crucial for the survival of melanocytes as well as melanoma cancer cells. In melanoma, the so-called "rheostat model" of MITF expression was established. According to the model, within tumors, there are subpopulations of melanoma cells with distinct MITF levels and cellular characteristics. Tumor cells expressing high levels of MITF are associated with differentiation and increased proliferation and low invasiveness, melanoma cells with low levels of MITF are more invasive and their proliferation is decreased. **Our aim was to regulate MITF expression in melanoma cells and monitor changes in proliferation, differentiation, invasiveness and expression of MITF target genes, EMT genes and CSC markers.**

6/ GLI2 expression was described to be inversely correlated with the expression of MITF in melanomas. **Therefore, the results obtained in the "rheostat study" would be discussed from the point of view that GLI2 negatively correlates with MITF expression level.**

3 METHODS

Here I present the list of methods, which I personally worked with. Methods are in detail described in published papers.

Cell culturing - see Publication I (p.37), II (p.47); III (p. 60-61) and IV (p.68)

Cloning plasmids, promoter-reporter constructs, expression constructs, site-directed mutagenesis -see Publication I (p.38)

Colony outgrowth assay, growth curves -see Publication II (p.47), III (p.61), IV (p.68)

Chromatin immunoprecipitation - see Publication I (p.38)

Detection of apoptosis - detection of apoptotic nuclei, TUNEL assay – see Publication II (p.47), III (p.61,62)

Flow cytometry – see Publication II (p. 47), III (p. 61)

Immunofluorescence microscopy – see Publication I (p.38), IV (p.68)

Invasivity assay – see Publication IV (p.69)

shRNA knock-down, production of lentivirus, lentiviral infection – see Publication IV (p.68)

Statistical analysis – see Publication I (p.38), II (p.48), III (p.62), IV (p. 69)

Transfections, promoter-reporter assays – see Publication I (p.38), II (p.47), III (p.62),

Viability assay – see Publication II (p.47), IV (p.69)

Western blotting – see Publication I (p.37), II (p.47), III (p.61), IV (p.68)

Wound healing assay - see Publication IV (p.69)

4 RESULTS AND DISCUSSION

Published research articles and author's contribution:

1/ **Vlčková, K*.**, Ondrušová, L., Vachtenheim, J., Réda, J., Dundr, P., Zadinová, M., Žáková, P., & Poučková, P. (2016). **Survivin, a novel target of the Hedgehog/GLI signaling pathway in human tumor cells.** *Cell Death and Disease*, *7*(1), e2048.
doi: 10.1038/cddis.2015.389
$IF_{2016} = 5{,}965$

Kateřina Kreisingerová (*Vlčková is maiden name) performed most of the experiments and is co-author of the manuscript

2/ **Vlčková, K.**, Réda, J., Ondrušová, L., Krayem, M., Ghanem, G., & Vachtenheim, J. (2016). **GLI inhibitor GANT61 kills melanoma cells and acts in synergy with obatoclax.** *International Journal of Oncology*, *49*(3), 953–960.
doi: 10.3892/ijo.2016.3596.
$IF_{2016} = 3{,}079$

Kateřina Kreisingerová performed most of the experiments and is co-author of the manuscript

3/ Réda, J., Vachtenheim, J., **Vlčková, K.**, Horák, P., & Ondrušová, L. (2018**). Widespread expression of hedgehog pathway components in a large panel of human tumor cells and inhibition of tumor growth by GANT61: Implications for cancer therapy**. *International Journal of Molecular Sciences*, *19*(9).
doi: 10.3390/ijms19092682.
$IF_{2018} = 4{,}183$

Kateřina Kreisingerová performed GANT61 experiments.

4/ **Vlčková, K.,** Vachtenheim, J., Réda, J., Horák, P., & Ondrušová, L. (2018). **Inducibly decreased MITF levels do not affect proliferation and phenotype switching but reduce differentiation of melanoma cells.** *Journal of Cellular and Molecular Medicine*, *22*(4), 2240–2251

doi: 10.1111/jcmm.13506

$IF_{2018} = 4,658$

Kateřina Kreisingerová performed most of the experiment and is co-author of the manuscript

Published review article (without impact factor):

5/ Kreisingerová K, Ondrušová L., Horák P, Vachtenheim J. (2020) Význam aberantně aktivované dráhy Hedgehog/Gli pro nádorovou progresi. *Klinická Onkologie,* 33(3):177-183.

doi: 10.14735/amko2020177

Kateřina Kreisingerová wrote the main part of the manuscript.

This publication is not reprinted in thesis, because I did not obtain the permission from the editorial board of Klinická Onkologie before the date of submission of doctoral thesis. The publication is available online at:

https://www.linkos.cz/files/klinicka-onkologie/471/5741.pdf

4.1 PUBLICATION I

Survivin, a novel target of the Hedgehog/GLI signaling pathway in human tumor cells

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Survivin, an important antiapoptotic protein, is expressed in tumors, whereas in normal tissues the expression of this protein is extremely low, defining a role for survivin as a cancer gene. Survivin exhibits multifunctional activity in tumor cells. However, why survivin expression is sharply and invariably restricted to tumor tissue remains unclear. Here, we identified 11 putative consensus binding sites for GLI transcription factors in the survivin promoter and characterized the promoter activity. Inhibitors of the Hedgehog/GLI pathway, cyclopamine and GANT61, decreased the promoter activity in reporter assays. ΔNGLI2 (which lacks the repressor domain) was the most potent vector in activating the survivin promoter–reporter. Moreover, GANT61, a GLI1/2 inhibitor, repressed endogenous survivin protein and mRNA expression in most cells across a large panel of tumor cell lines. Chromatin immunoprecipitation showed GLI2 binding to the survivin promoter. The ectopic GLI2-evoked expression of endogenous survivin was observed in normal human fibroblasts. GANT61 decreased survivin level in nude mice tumors, mimicking the activity of GANT61 in cultured cells. The immunohistochemistry and double immunofluorescence of human tumors revealed a correlation between the tissue regions showing high GLI2 and survivin positivity. Thus, these results demonstrated that survivin is a classical transcriptional target of GLI2, a Hedgehog pathway signaling effector. This potentially reflects the high expression of survivin in human tumor cells. As the Hedgehog pathway is upregulated in virtually all types of cancer cells, these findings substantially contribute to the explanation of uniform survivin expression in tumors as a potential target for the development of a more effective treatment of cancers through the inhibition of GLI2 to restrain survivin activity.

Cell Death and Disease (2016) 7, e2048; doi:10.1038/cddis.2015.389; published online 14 January 2016

Survivin is a single-baculovirus IAP repeat protein that plays a role in multiple processes, including proliferation and cell survival. Survivin is abundantly and ubiquitously expressed during development[1] and this expression is consistently recapitulated in tumor tissue. The expression of this protein has been associated with the aggressive biological features of tumors, resistance to radiation and chemotherapy and poor clinical outcome.[2] Since its discovery in 1997,[3] the mechanism that maintains high survivin expression in tumors and absent or extremely low survivin expression in normal tissues remains unknown. It has been suggested that the basal transcription of the survivin gene is primarily regulated through the Sp family of transcription factors.[4,5] Although binding sites for several pro-oncogenic transcription factors (Sp1, STAT3, NF-kB, KLF5, E2F1, DEC1 or TCF) are present in the survivin promoter (reviewed by Boidot et al.[6]) and might be important in the elevation of survivin levels, specifically in individual tumors, it is unlikely that these sites could guarantee the high survivin levels observed in all malignant cells. For example, KLF5 increased the resistance of ovarian cancer cells to drug treatment,[7] and DEC1 increased the expression of survivin via Sp1 sites in kidney and lung adenocarcinomas.[8] A recent study reported that HH/GLI via GLI1 and GLI2 transcription factors activated the E2F1 promoter in melanoma cells, and

E2F1 contributed to the increase of melanoma cell growth,[9] constituting a positive feedback loop.

The canonical activation of the Sonic Hedgehog (HH/GLI) cascade involves the binding of the ligand (Shh) to the 12-pass membrane protein PATCHED (PTCH), which releases the activity of adjacent 7-pass transmembrane protein Smoothened (SMO). Subsequently, the effector proteins GLI(1–3) are released from the inhibitor SuFu (Suppressor of Fused) and translocated to the nucleus to activate target genes.[10] HH/GLI is important during normal embryonic development, and the aberrant activation of this signaling pathway has been associated with many human cancers. The activation of HH/GLI increases proliferation and survival, induces cancer stem cell marker expression and enhances bone metastasis.[11,12]

Several studies have implicated a non-canonical Hh signaling pathway in regulating HH/GLI signaling, thus substituting the necessity of upstream ligand signaling.[13–16] Several pathways, such as AKT,[17–19] MAPK,[18] RAS[20] or EGFR,[21,22] can activate GLI factors in tumor cells. Conversely, many critical cellular pathways, such as proliferation, DNA damage repair, apoptosis, autophagy, epithelial–mesenchymal transition (EMT), telomerase activity, invasion, metastasis and maintenance of tumor stem cells, are regulated through

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Abbreviations: DEC1, differentiated embryo-chondrocyte 1; HH/GLI, Hedgehog–GLI signaling pathway; GLI, glioma-associated oncogene homolog; KLF5, Kruppel-like factor 5; Shh, sonic hedgehog; STAT3, signal transducer and activator of transcription 3
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GLI DNA binding sites in survivin promoter	Sequence	Position in survivin promoter
GLI consensus DNA binding site 1	GACCACCCA	
GLI consensus DNA binding site 2	TGGGTGGTC	
GLI DNA binding site 1	AACCACCCA	-1781 to -1773
GLI DNA binding site 2	TGGGTGAAG	-1523 to -1515
GLI DNA binding site 3	TGGGAGGCC	-1417 to -1409
GLI DNA binding site 4	GACCAGCCT	-1374 to -1366
GLI DNA binding site 5	CCCCACCCT	-812 to -804
GLI DNA binding site 6	TGGGTGCAC	-203 to -195
GLI DNA binding site 7	GACCACGGG	-191 to -183
GLI DNA binding site 8	GCACACCCC	-149 to -141
GLI DNA binding site 9	GACCGCCTA	-100 to -92
GLI DNA binding site 10	TGGGTGCCC	+2 to +10
pGLI DNA binding site 11	GACCACCGC	+46 to +54
Mutated sequence of GLI DNA binding sites (1)	ATCGCGAAT	
Mutated sequence of GLI DNA binding sites (2)	ATTCGCGAT	

Figure 1 Overview of the GLI-binding sites and their position in the human survivin promoter. Numbering is from initiating ATG (+1). Full consensus sites and the sequence used for introduced mutations is also given. Black letters are consensus nucleotides, light letters are non-consensus nucleotides. Survivin-containing GLI sites are degenerated in 1–3 nucleotides. GLI site positions are shown in Figure 2

GLI transcription factors.[23–27] In recent years, a specific and effective GLI1/2 inhibitor GANT61 has been successfully used for the *in vitro* and *in vivo* treatment of cancer cells containing either the canonical or non-canonical activation of HH/GLI.[28–29]

Here, we show that survivin is a transcriptional target of the Hedgehog pathway effector factor GLI2, and harbors 11 potential GLI-binding sites in the promoter. GLI2 is a pro-invasive protein present in most tumor cell lines and this protein could substantially contribute to the stably elevated survivin levels observed in tumors. We further demonstrated the binding of GLI2 to the survivin promoter and the decreased expression of survivin protein and RNA after treatment with GLI2 inhibitor GANT61 in a large panel of tumor cell types. Furthermore, endogenous survivin expression is evoked through the ectopic expression of GLI2 in normal human fibroblasts. Overall, the results of the present study suggest that survivin is a novel target of the Hedgehog/GLI pathway and GLI2 is the primary upregulating factor for this protein. Thus, the maintenance of deregulated survivin expression in many tumors could reflect activated Hedgehog pathway.

Results

Activities of the survivin promoter–reporter with 11 potential GLI-binding sites determined in A549 cells. We reasoned that the high expression of the cancer protein survivin, invariably present in all tumor cells, should have a more significant impact than anticipated. The presence of several pro-oncogenic sites in the promoter presumably cannot explain the universal expression of this protein in tumors (for promoter map, see Boidot *et al*.[6]). We observed that the survivin promoter contains 11 sites for binding GLIs, effectors of the HH/GLI signaling pathway; however, none of these sites are full consensus sequences (Figure 1). Non-consensual sites with two or three mismatches can still activate the transcription of other GLI-regulated genes, such as BCL2 and osteopontin.[30,31] Thus, the survivin promoter

contains attributes for the binding and eventual activation by GLI transcription factors.

To determine the activity of the survivin promoter and elucidate the importance of the GLI sites, we transfected several versions of the promoter into A549 cells and measured luciferase activity (Figure 2). The proximal promoter (−990 to −10) was sufficient to activate luciferase activity and was only slightly less efficient than the longer (−1814 – 10) promoter. The distal portion of the promoter contains four GLI sites (no. 1–4), and the mutations at these sites appreciably decrease promoter activity (~2.5-fold; Figure 2). We individually mutated all 11 sites, but no single mutation (with the exception of site no. 10) had a significant effect on the promoter activity (data not shown). Intriguingly, after the addition of a short downstream region (−10 to +57 nt) containing sites 10 and 11, the strong repression of promoter activity appeared. However, when site 10 was mutated the activity was reverted to normal, indicating that the site 10 is an inhibitory site. Of note, as survivin is widely expressed in tumors, this site cannot be necessarily repressory in the genomic context, where transcription occurs differently than in reporter assays.

To further precisely define the regulatory regions of the survivin promoter, we deleted the central region of the proximal promoter (−390 to −60) (Figure 2). After the deletion of this region in both the longer and shorter survivin promoters, the activity was completely abrogated. Because this region comprises all Sp sites, this result is consistent with the known role of Sp1 and Sp3 as factors necessary for the basic promoter activity.[4,5] The role of GLI sites no. 6–9 in the deleted region cannot be precisely established because some of these areas overlap the Sp sites. Taken together, these results suggest that the promoter and regulation of survivin through GLI sites is complex, with one site (no. 10) being clearly inhibitory in reporter assays, and the proximal region, containing four GLI sites, and the central region with Sp sites (−390 to −60) are required for the transcriptional activity of the promoter. Most likely, a specific combination of some of the sites is responsible for the full reporter activity. Moreover, the four distal sites increase survivin promoter activity.

The survivin promoter–reporter is inhibited through Hedgehog/GLI inhibitors cyclopamine and GANT61 and specifically activated through ΔNGLI2. Given that many GLI sites are present in the survivin promoter, inhibitors of the HH/GLI pathway should suppress promoter activity. Indeed, a SMO inhibitor cyclopamine significantly diminished the activity of the survivin promoter in many cell lines to various extents (30–70%), as exemplified in three melanoma cell lines (Figure 3a). We next assessed the inhibition of HH/GLI using a downstream inhibitor GANT61, which specifically inhibits GLI1/2 activity. In eight tested cell lines, GANT61 consistently inhibited the promoter, with only 70–20% of the original activity remaining (Figure 3b). The effect of cyclopamine and GANT61 was dose dependent (Supplementary Figure S1). These results suggest that survivin expression is regulated through the HH/GLI pathway.

To further confirm the transcriptional regulation of survivin through HH/GLI signaling, we compared the activity of four types of survivin promoters with the known GLI targets, the
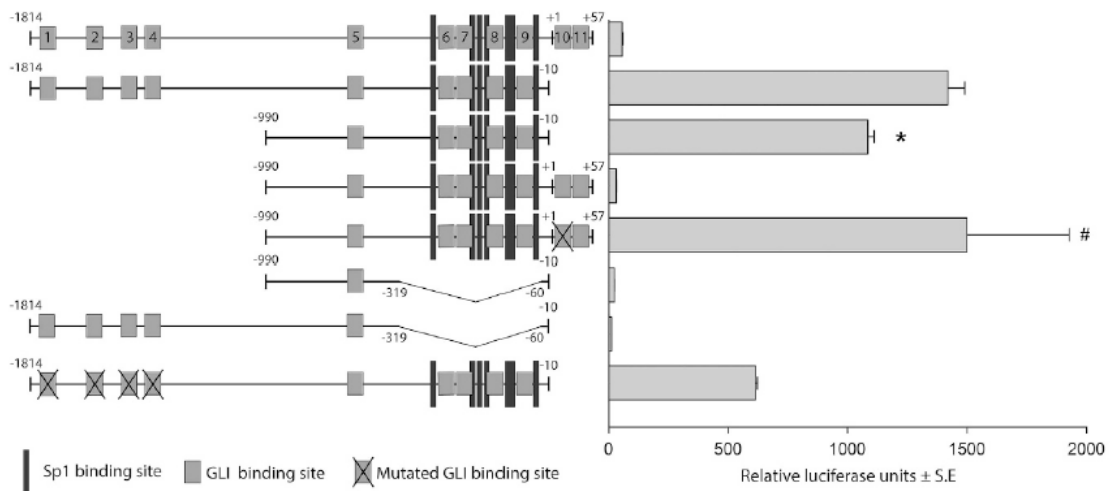
Figure 2 Schematic representation of the survivin promoter with the numbering (relative to translation start) and indicated GLI-binding sites (no. 1–11). The GLI sites correspond to those depicted in Figure 1. The positions of Sp-binding sites are also designated. To determine the luciferase values, the promoters cloned in pGL3basic (Promega) were transfected into A549 cells by using LipoJet (SignaGen) (1 μg per well in a 12-well plate). Dual luciferase system (Promega) was utilized for detection. Each sample was transfected in triplicates and three repeated experiments were performed, in which similar results were obtained. S.E. values are shown for all luciferase results. *, statistically significant ($P < 0.05$); #, statistically not significant (related to the $-1814 - 10$ promoter)

12xGLI promoter[32] and the PATCHED promoter.[33] An empty vector (control) or expression FLAG-tagged vectors for GLI1, GLI2, ΔNGLI2 (an active form of GLI2)[34], GLI3 and GLI3Δ (106–236) were cotransfected into A549 cells. Among all promoters, ΔNGLI2 exhibited the highest activation potential. The super-GLI promoter 12xGLI was activated nearly 100-fold and the PATCHED promoter 16-fold through ΔNGLI2 (Figure 3c, upper row). The survivin promoters were stimulated seven- to ninefold (Figure 3c, lower row). GLI1 and GLI3 did not activate the survivin promoters. GLI3 is a known repressor of some HH/GLI-regulated genes.[35,36] Stimulation through GLI3Δ(106–236), which lacks the repressor domain, was consistently higher than that of GLI3 (Figure 3c, lower row). The most prominent activation of all promoters was through ΔNGLI2. The activation by GLI2, ΔNGLI2 and GLI3Δ (106–236) were dose dependent (Supplementary Figure S2). All GLI proteins were expressed, as assessed by the anti-FLAG antibody (Figure 3d).

GANT61 inhibits survivin protein and mRNA expression in tumor cells. Instead of studying the HH/GLI target survivin in a specific tumor, we used a general approach to determine a potentially more common role for GLIs in endogenous survivin expression in several tumor cell types. The consequence of GLI factors inhibition was estimated across a large panel of cancer cell lines (total 40 lines, listed in Supplementary Table S1). Although approximately one-half of the cell lines were melanomas or small-cell lung carcinomas, several other malignant cell types, such as NSCLC, colon and pancreatic cancers, were also represented. Western blot analysis revealed that GANT61 inhibited or attenuated survivin expression in most cell lines (Figure 4a) in a dose-dependent manner. GANT61 remained on the cells for 24 h at 0, 10 or 20 μM and the RIPA extracts were analyzed. Marked differences in the extent of decreased

survivin expression were observed. In some cell lines survivin expression was dramatically decreased (SK-MEL-3, WM-35, SW13), particularly with 20 μM GANT61, and some cells responded weakly. There was no change in expression of the controls SRC and actin. The differences in the decreased survivin protein expression did not correlate with the type of tumor. In some cell lines, no or minimal changes in survivin protein expression were detected after GANT61 treatment (Supplementary Figure S3). Regl et al.[30] demonstrated that BCL2 transcription is activated through GLI2. In the present study, we observed that BCL2 was down-regulated through GANT61 in only one cell line, SK-MEL-28 (Figure 4a). Osteopontin (OPN) has previously been identified as a target of HH/GLI signaling in melanoma cells.[31] In the present study, however, we did not detect a decrease in OPN expression after GANT61 treatment in any cell line (data not shown).

Consistently, real-time PCR revealed that the inhibition of survivin protein levels was accompanied with the down-regulation of survivin mRNA to various extents (Figure 4b), confirming the predominantly transcriptional repression of this gene after GANT61 treatment (both parameters correlated, albeit weakly; Supplementary Figure S4). We also estimated correlation of quantified values of GLI2 and survivin proteins from western blots, but these two parameters did not correlate (Supplementary Figure S5). This is not surprising because GLI2 has many targets in the cell and both low and high survivin protein levels were responsive to GANT61. In some cell lines (e.g., SK-MEL-3), the decline in RNA levels was apparently lower than the decrease in protein levels (Figure 4a). We hypothesized that the rate of survivin protein degradation might also be accelerated or delayed in some cell lines. Thus, four cell lines were randomly selected and incubated with GANT61 for 24 h and MG132 (MG), a proteasome inhibitor, was added to one sample for the last

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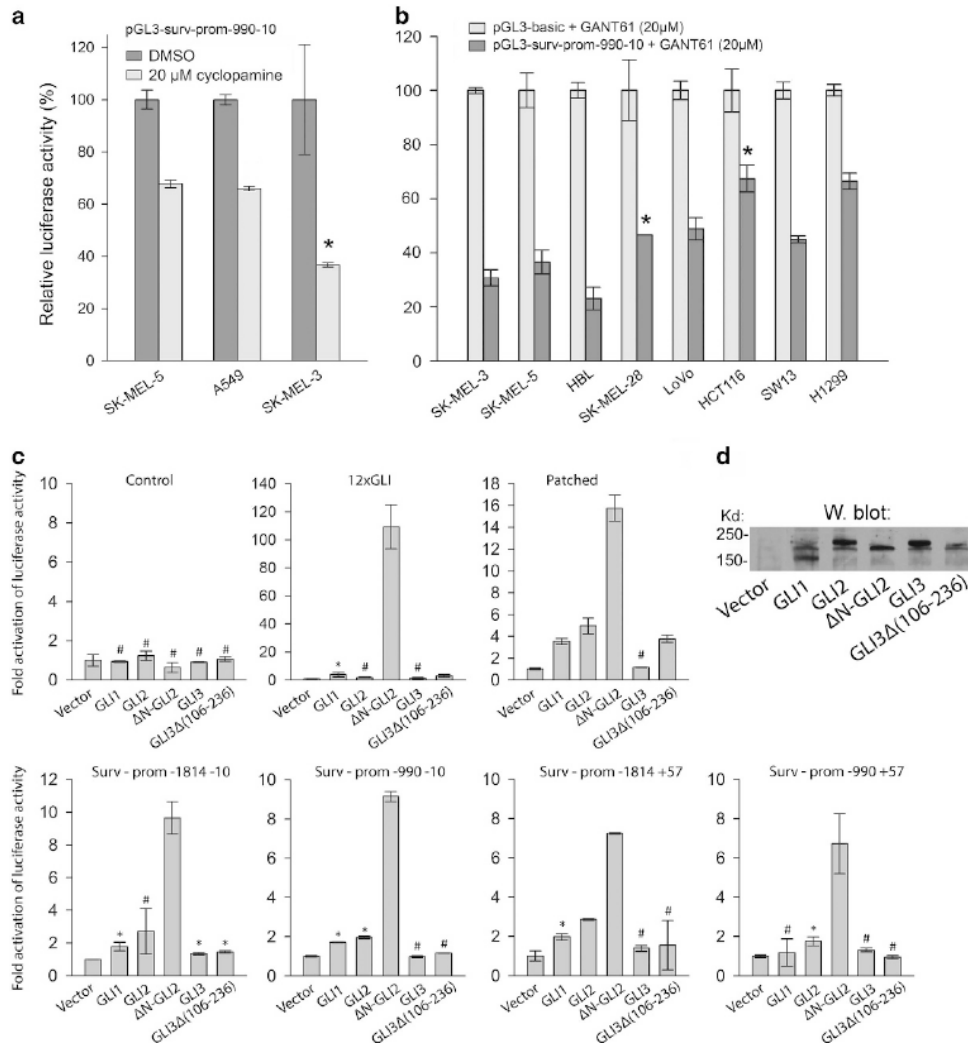
Figure 3 Inhibition of survivin promoter by cyclopamine and GANT61 and survivin promoter stimulation by GLI factors in A549 cells. (a) Cyclopamine inhibits luciferase activity in the three melanoma cell lines after 24 h treatment. (b) Survivin promoter activities are inhibited after 24 h incubation with GANT61 in eight tumor cell lines. GANT61 has a minimal effect on control promoter (usually <5%). Therefore, control values are set as 100% (control plasmid with GANT61). (c) Stimulation of several promoters with GLI1, GLI2, ΔNGLI2, GLI3 and GLI3Δ (106–236). Upper row: the activities of the control promoters are similar, an artificial 12xGLI promoter and a known GLI target PATCHED promoter are strongly activated by ΔNGLI2. Lower row: the four indicated survivin promoter versions are activated, albeit sometimes weakly, by all three GLIs, maximally by ΔNGLI2 (from 7- to 10-fold). GLI3 displayed no activation of survivin promoters, while higher activity was noted with GLI3Δ(106–236). Please note that the control values of survivin promoters are set as 1 and the amount of their DNA is fivefold lower than in Figure 2, but the controls still have relatively high activity, as documented in RLU in Figure 2. Each experiment was repeated at least two times (d) Western blot demonstrating the expression of GLI factors expressed in (c). All GLI proteins were N-terminal FLAG-tagged constructs and detected through the anti-FLAG antibody

6 h to prevent survivin degradation (Supplementary Figure S6). In SK-MEL-3 cells (high RNA), MG did not overcome the diminution of the GANT61-mediated survivin protein level, indicating that some GLI factors also protect survivin protein from degradation. In A427 cells, the decreased survivin levels reflect decreased RNA expression. The 501mel cells (high RNA) reacted poorly to GANT61 treatment and were not influenced after incubation with MG. However, in H1299 cells, MG increased the final levels of survivin protein, indicating that the protein protection is mediated through an as yet unknown,

GLI-independent mechanism. Taken together, these results suggest that the decrease in endogenous survivin in the presence of GANT61 is predominantly a transcriptional event, whereas the specific cellular context might also differently modify protein degradation.

GLI2 binds to the survivin promoter in cells. Next, to confirm the direct interaction of GLI2 with the endogenous survivin promoter, we performed a quantitative chromatin immunoprecipitation assay (ChIP) in A549 cells using two different
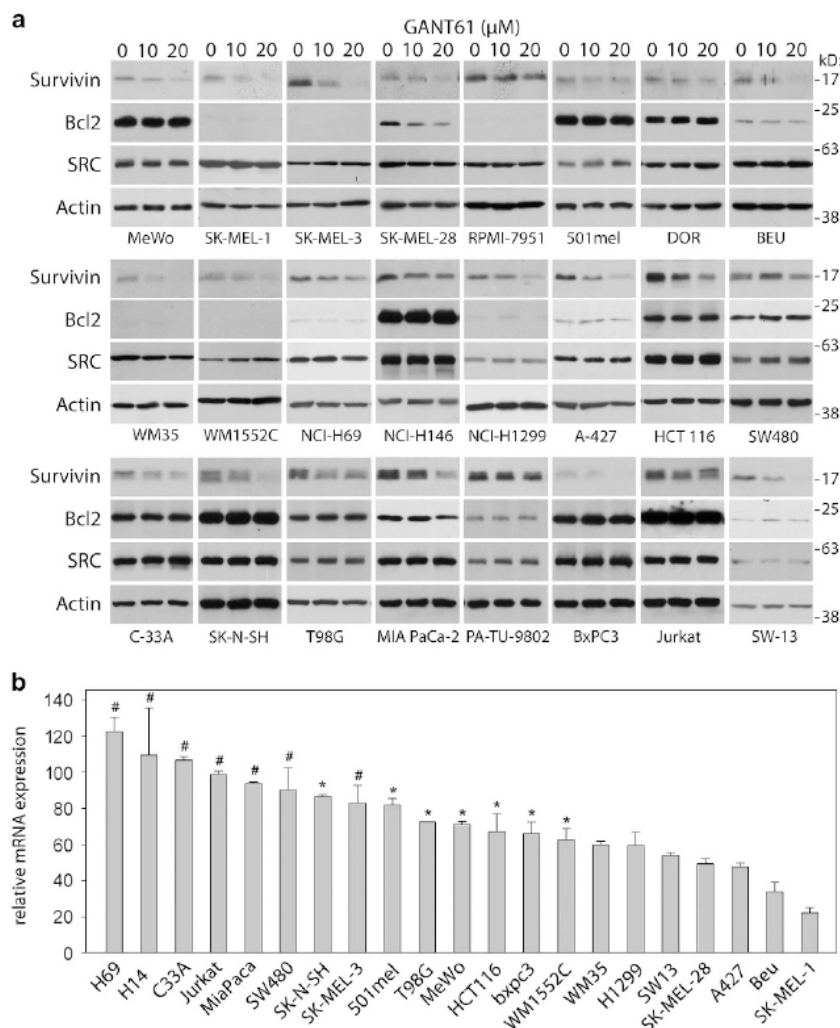
Figure 4 Inhibition of the survivin cellular protein and its mRNA expression after GANT61 treatment. (a) Cells were treated with indicated GANT61 concentrations for 24 h. RIPA buffer extracts were prepared, blotted and probed with the indicated antibodies. Additional cell lines with no or only minute changes of survivin protein expression are shown in Supplementary Figure S3 (b) Real-time PCR experiments performed from controls and samples inhibited by 20 μM GANT61. After 24 h in GANT61, the RNA was isolated with Trizol, reverse transcribed and used in qPCR. Distinct decrease of RNAs levels was observed relative to controls (0 μM GANT61). RNA diminutions do not correlate neither with the tumor cell type nor with the proliferation rate. Some tumor cells produced only slightly reduced RNA, whereas the survivin protein diminished strikingly (e.g., SK-N-SH and SK-MEL-3), suggesting that the protein degradation was retarded as well (see text and Supplementary Figure S6). Knockdown of GLI2 RNA by shRNA plasmid constructs in MeWo and SW13 cell lines also diminished survivin mRNA and protein expression (not shown). #, not significant; *, significant at $P<0.05$; not marked, significant at $P<0.01$ (relative to 100%)

anti-GLI2 antibodies (Figure 5a). Preliminary experiments in several cell lines revealed only low (about twofold) enrichment of GLI2 on the promoter. Therefore, the ΔNGLI2 expressing plasmid was transfected into A549 cells, and after performing ChIP experiments, the GLI2 enrichment on the promoter was quantified using SYBR green real-time PCR. The results confirmed the prominent enrichment supporting that GLI2 was indeed recruited to the survivin promoter in cells. Similar qPCR results were obtained with two different amplifications resulting in two distinct amplicons (Figure 5a, left and right).

Ectopic GLI2 elicits endogenous survivin expression in normal human fibroblasts. To further demonstrate that the survivin promoter is an actual GLI2 target in living cells,

expression vectors for GLI1, GLI2 and ΔNGLI2 were transfected into the normal diploid human fibroblast cell line IMR90 which does not express survivin or GLI2. Western blotting clearly detected survivin in the resulting cell lysates. The strongest signal was obtained with the most potent activator ΔNGLI2 (Figure 5b), suggesting that GLI2 is a direct survivin promoter activator in cells. Ectopic GLI2 has been properly expressed (not shown).

GANT61 partially inhibits tumor growth in GANT61-sensitive melanoma cell xenografts. *In vitro*, GANT61 completely killed SK-MEL-3 cells in 4 days, whereas 501mel cells were much more resistant (Supplementary Figure S7e). To determine whether the *in vitro*

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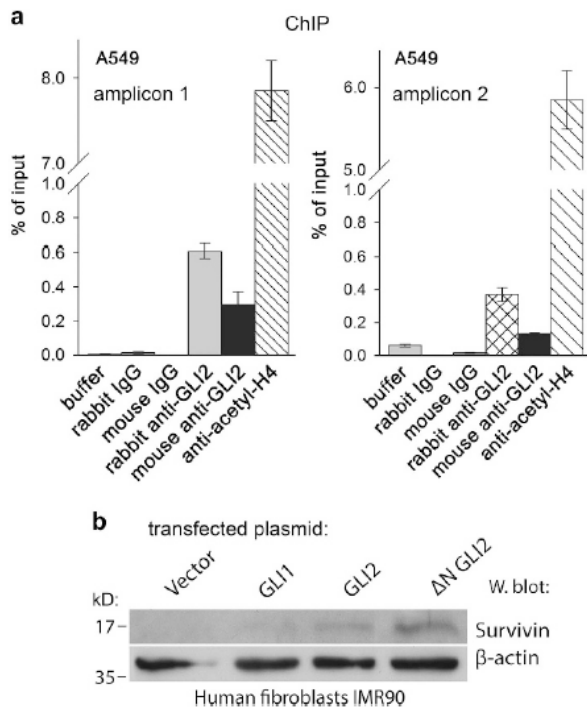
Figure 5 Chromatin immunoprecipitation and endogenous survivin induction by GLI in IMR90 fibroblasts. (a) A549 cells transfected with ΔNGLI2 expression vector were chromatin immunoprecipitated and resulting DNA was subjected to quantification by qPCR. All negative controls (buffer, mouse or rabbit non-immune IgG) and a positive control (antibody against acetylated histone H4) were included. Significant enrichment of GLI2 samples was evident in both amplifications. The two real-time PCRs differed only in the antisense primer. The amplicon shown in (a) on the right was shorter. (b) Transient transfection of GLI1, GLI2 and ΔNGLI2 into human IMR90 fibroblasts, which normally do not express survivin and GLI2, induced expression of survivin protein (about 30% cells were transfected, not shown). Most prominent expression of survivin protein was achieved with ΔNGLI2, which was also most active in reporter assays (Figure 3). Western blot with the anti-GLI2 antibody verified the GLI2 increase in transfected cells. The results indicate the dependence of survivin expression on GLI2 in normal cells

effects of GANT61 are recapitulated *in vivo*, SK-MEL-3 and 501mel cells were subcutaneously engrafted into athymic nude mice. While the tumor masses with GANT61 remained as controls in 501mel cells (Supplementary Figure S7b), two SK-MEL-3 tumors were markedly reduced (the size of one tumor deviated and did not diminish; Supplementary Figure S7a). Expectedly, in all tumors from GANT61-treated animals, survivin expression estimated by Western blot was markedly reduced (with the exception of the smallest 501mel tumor) (Supplementary Figures S7c and d). The immuno-chemistry of animal tumors showed positive survivin staining in controls, while only scarce positive cells remained in tumors from treated animals (Supplementary Figure S7f). Growth curves are also shown for all tumors (Supplementary Figures S7g and h).

The immunochemically positive GLI2 and survivin regions in human tumor sections overlap and colocalize by immunofluorescence. Additional tests were performed to validate the role of GLI2 in survivin expression. If GLI2 is

an activator of survivin transcription in cell models, then the positive areas for both proteins should correlate in authentic human tumor sections. We examined parallel sections of 35 randomly selected human tumors (lung carcinomas, ovary and tubal carcinomas and melanomas) and examined the GLI2 and survivin staining. Indeed, positively stained GLI2 areas correlated with survivin-positive areas in all tumors, although some regions of GLI2 positivity with survivin negativity and vice versa were also observed (not shown). The statistically significant positivity correlation was observed only in the highest score (4). Some tumor cells showed perfectly correlated positivity for both proteins in parallel sections, as exemplified in two cases of lung adenocarcinomas (Figure 6b). Apparently, strongly positive GLI2 cells were observed in parallel with sections showing positive survivin staining. To substantiate these results, double immunofluorescence was performed demonstrating colocalization of survivin and GLI in strongly staining areas in tumors. The simultaneous positivity of GLI2 and survivin images were highly statistically significant (Figure 6a), supporting the IHC results.

Thus, these data showed the overall correlation between GLI2 and survivin staining in tumors, strongly supporting that the HH/GLI pathway is an activator of survivin expression.

Discussion

The Hedgehog pathway is important for multiple tumor types, although this signaling pathway was initially suggested as necessary for only basal cell carcinoma and medulloblastoma.[37] Additionally, aberrant HH/GLI signaling plays a critical role in commonly occurring tumors, such as non-small-cell lung cancers[38] and many others.[39] However, the common mechanism for the maintenance of the high survivin levels in tumors remained obscure.

Several genes important for cancer growth are regulated through GLI factors. Previous studies have demonstrated the regulation of BCL2 via the HH pathway through GLI1,[40,41] while other studies have reported BCL2 activation through GLI2.[30] Bar *et al.*[42] reported that HH/GLI-activated BCL2 was important for the survival of medulloblastoma (a cancer not examined in the present study). However, in the present study, we observed BCL2 inhibition through GANT61 in only one cell line (Figure 4a). Conceivably, BCL2 might represent a HH/GLI target pivotal for tumor growth only in specific cell lines. Notably, even Sp factors, important for basal survivin promoter function, have also demonstrated pro-tumorigenic activity.[43]

The HH/GLI pathway functions in a paracrine and autocrine manner. Some inhibitors of HH/GLI have contributed to the downregulation of the pathway targets in the stromal micro-environment, suggesting that the effect on HH/GLI signaling is dependent on the stroma and a paracrine signaling mech-anism.[44] Owing to the critical role of this signaling pathway in general tumor maintenance, intensive clinical studies utilizing several HH/GLI inhibitors have been performed.[39,45]

Here, we revealed a general mechanism resulting in survivin expression in cell lines. More than half of the cell lines analyzed showed downregulated survivin expression after treatment with 20 μM GANT61, and several lines manifested nearly complete inhibition (Figure 4a). Further,
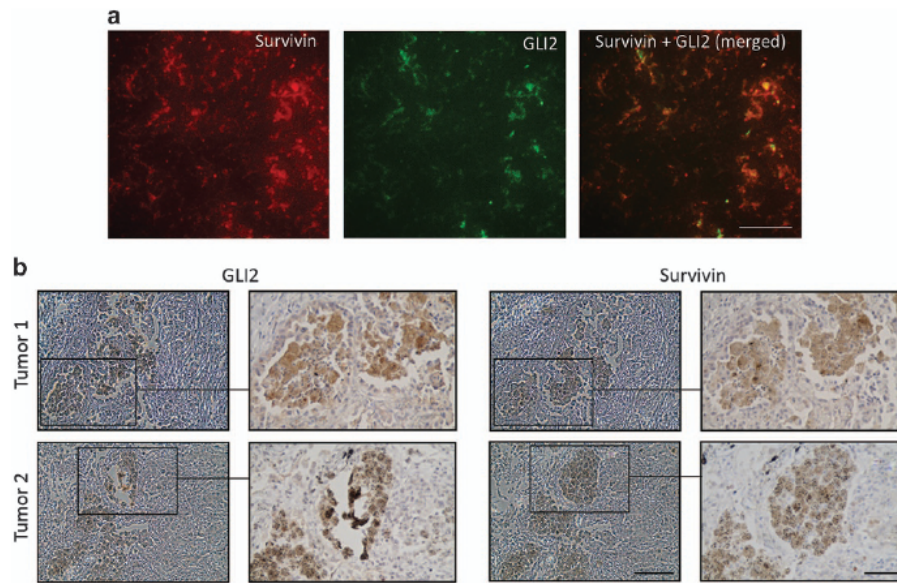
Figure 6 Immunohistochemical and immunofluorescence staining of GLI2 and survivin in human tumors. (a) Double immunofluorescence with anti-GLI2 (rabbit) and anti-survivin (mouse) antibodies demonstrating colocalization of both proteins in the areas of high staining activity of both. The staining has been performed on the frozen tissue (ovarian tumor) after acetone fixation. High correlation has been found using the FIJI software (Spearman's rank correlation value: 0.882). Bar represents 20 μm. (b) Examples of two areas of lung tumors showing the highest score (4) of staining for survivin and GLI2. Parallel sections were stained. The same stained structures are clearly recognized in both lower and higher magnifications. Bars, 100 and 50 μm (sectors)

higher doses of GANT61 efficiently inhibited survivin protein expression (data not shown), indicating the dose-dependent decrease and the reliance of survivin level on HH/GLI. Whereas some cells were resistant to the GANT61-mediated reduction of survivin expression (Supplementary Figure S3), other mechanisms likely maintain the survivin level observed in tumor cells. The survivin protein levels of GANT61-treated cells correlated with the real-time PCR values in samples where real-time PCR was performed (Supplementary Figure S4).

We dissected and mutated the survivin promoter, revealing many GLI-binding sites, and demonstrated that the promoter–reporter is substantially inhibited through the HH/GLI inhibitors cyclopamine and GANT61 (Figures 1–3). The large number of GLI-binding sites made it difficult to precisely determine the combination of sites critical for survivin activity mediated via HH/GLI. We propose that GLIs are associated with the promoter in each tumor cell and other factors dictate survivin expression. We previously demonstrated epigenetic mechanism important for survivin expression. The knockdown of BRG1, an ATPase of the SWI/SNF chromatin remodeling complex, dramatically reduced the expression of survivin RNA and protein in melanoma cells.[46]

Notably, both survivin and HH/GLI signaling are active during development. Survivin is lethal in homozygous knock-out mice,[47] similar to several components of the HH/GLI pathway (reviewed by Yang et al.[39]). Consistent with the present study, Brun et al.[48] revealed that survivin is a critical therapeutic target in medulloblastoma cells, where HH/GLI signaling in invariably increased. The inhibition of survivin expression through the specific inhibitor YM155 profoundly affected the viability of tumor cells and sensitized tumors to radiation.

Taken together, we revealed the mysterious manner of expression of the tumor protein survivin, although the described mechanism is not functional in all tumor cell lines. These results show the direct activation of survivin expression through the HH/GLI signaling mediated by GLI2. Importantly, anti-GLI2 therapy or combined anti-GLI2 and anti-survivin therapies might decrease survivin in tumors and markedly improve the treatment of cancer.

Materials and Methods

Cell lines and treatments. We utilized 40 tumor cell lines of various origin (listed in Supplementary Table S1). Cell lines were cultivated in the cultivation media as recommended with 10% FCS and antibiotics. Most cell lines were from American Type Culture Collection (Manassas, VA, USA). Cell lines DOR, BEU, HBL, 501mel and PA-TU-8902 were from other sources (Supplementary Table S1). The cell lines were treated with inhibitors as indicated in the figures and figure legends (the final concentrations were 20 μM GANT61 and 20 μM cyclopamine).

Quantitative real-time PCR. Total RNA was isolated using TRIZOL (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (2 mg) was reverse transcribed using the Super Script II reverse transcriptase (Life Technologies). Quantitative PCR (qPCR) was conducted using Taqman system QuantiTect Probe PCR Kit (Qiagen, Hilden, Germany) on ViiA7 Real-Time PCR system (Life Technologies) according to the manufacturer's instructions. Similar results were obtained in two independent experiments. The qPCR primers for survivin were: forward, 5′-AAGAACTGGCCCTTCTTGGA, reverse, 5′-CAACCGGACGAATGCTTTT, probe, 5′-6-FAM-CCAGATGACGACCC CATAGAGGAACA-TAMRA. Actin was used as an internal standard control.

Cells extracts, western blots and antibodies. To prepare whole-cell extracts for immunoblotting analysis, cells were lysed in RIPA buffer (1% NP-40, 150 mM NaCl, 5 mM EDTA, 0.5% sodium deoxycholate, 50 mM Tris-HCl pH 7.5, 0.1% SDS) with added protease and phosphatase inhibitors 1 mg/ml leupeptin, aprotinin and pepstatin, COMPLETE (Roche Diagnostic, Mannheim, Germany) was added as recommended by the supplier. The buffer was supplemented with

PhosStop (Roche) and 1 mM phenylmethylsulfonylfluoride. Equal amounts of protein were loaded on 10–12% SDS-polyacrylamide gels and transferred onto PVDF membranes after electrophoresis. Blots were incubated with primary and horseradish peroxidase-conjugated secondary antibodies. Chemiluminescent detection was used. The antibodies for western blots: anti-survivin (D-8) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA), anti-SRC and anti-BCL2 were from Cell Signaling Technology (Danvers, MA, USA) and β-actin (AC-74) from Sigma-Aldrich (St. Louis, MO, USA). Anti-GLI2 (C-10) antibody was from Santa Cruz Biotechnology or Biorbyt (San Francisco, CA, USA). Anti-FLAG antibody (M2) was from Sigma.

Transfections, luciferase promoter-reporter assay, colony formation. Transient cell transfections of the promoter–reporters (Figures 2 and 3) were performed on 12-well plates by using transfection reagents LipoJet or PolyJet (SignaGen Laboratories, Rockville, MD, USA). The pRSV-Renilla luciferase expression vector was cotransfected to monitor transfection efficiency. pGL3basic vector (Promega, Madison, WI, USA) was used as a control promoter. Expression vectors were cotransfected as indicated in the figures. Cell lysate was used for dual luciferase assays (Promega) performed as recommended by the supplier's instructions on a Turner Designs 20/20 luminometer (Promega). Data were normalized to *Renilla* luciferase activity (internal control) as arbitrary units. The inactive compound structurally similar to cyclopamine (tomatidine) gave the same results as vehicle (not shown). Thus, only vehicle was used as a control in all experiments with cyclopamine. Statistical analysis of luciferase values was performed using a two-tailed unpaired Student's *t*-test. Colony outgrowth assays were carried out by seeding the cells in 12-well plates. After GANT61 treatment for the indicated time period the cells were stained by crystal violet.

Plasmids and site-directed mutagenesis. pGL3-PTCH1 was obtained from Prof. Aberger, Salzburg, Austria. 12xGLI-TK-Luc plasmid was a gift from Prof. R Toftgard, Karolinska Institutet, Sweden. All versions of the survivin promoter and its mutants have been cloned as *Xho*I–*Hind*III inserts in the pGL3basic plasmid. The following survivin promoter–reporter plasmids were generated (numbering is related to the start of translation, +1): − 1814+57, − 990+57, − 1814 − 10, − 990 − 10, − 1814 − 10Δ(−319 − 60) and − 990 − 10Δ(−319 − 60). Further, single-site mutants were prepared by two-step PCR mutagenesis using Phusion DNA polymerase (Fisher Scientific, Pittsburgh, PA, USA). Original GLI1 (GLI K12, #16419), GLI2 (pCS2-MT GLI2 FL, #17648), ΔNGLI2 (pCS2-MT GLI2 delta N, #17649) and GLI3 (GLI3 bs-2, #16420) were purchased from non-profit plasmid repository Addgene (Cambridge, MA, USA). Their coding sequences were amplified by PCR and cloned into the pcDNA3.1 expression vector or to the pFLAG-CMV-4 background to obtain FLAG-tagged GLI proteins for the use in experiments shown in Figure 3c. PCR was used for cloning all GLIs to the final plasmids. pCMV-Sp1 was obtained from Addgene (#12097), pCMV6-XL5-NfkappaB from Origene (Rockville, MD, USA). All final plasmids were verified by sequencing (GATC Biotech, Constance, Germany).

Chromatin immunoprecipitation. A549 cells were transfected with the pcDNA3-ΔNGLI2 expression plasmid. After 2 days cells were fixed with 1% formaldehyde, incubated with glycine solution and washed four times with PBS. The cell extracts were isolated and processed according to instructions of the ChIP-IT High Sensitivity Kit (Active Motif, Carlsbad, CA, USA). As a positive control, anti-acetylated histone H4 antibody was used (Millipore, Billerica, MA, USA). Negative controls were buffer, rabbit or mouse non-immune IgG. To detect GLI2 bound on the promoter, mouse anti-GLI2 (C-10) (Santa Cruz Biotechnology; sc-271786) and rabbit anti-GLI2 (Abcam, Cambridge, UK; ab26056) were used. For the detection of ChIP-generated DNA, real-time PCR was performed by the QuantiTect SYBR Green PCR Kit (Qiagen). The amplification has been performed with two alternative primer pairs. The primers used for the amplification were sense 5′-TTTGTCCTT CATGCCCGTCT, antisense 5′-TGTAGAGATGCGGTGGTCCT, or the same sense primer and a different antisense, 5′-GCGGGGCATGTCGGGA. Both amplifications gave similar results.

Tumor xenografts. To investigate whether the GANT61 regulates HH/GLI-induced growth *in vivo*, melanoma cell lines SK-MEL-3 and 501mel were engrafted subcutaneously into athymic nude mice (strain CD-1, 1 million of cells per single site). Cells were resuspended in Matrigel (Becton Dickinson, Franklin Lakes, NJ, USA) and inoculated in one site of the right lateral flank of 4- to 5-week-old female mice (Jackson Laboratories, Sacramento, CA, USA). Subcutaneous tumor size was measured three times a week with a caliper and tumor volumes were calculated. GANT61 was administered three times a week intratumorally for 2 weeks. After ending the experiment, the fresh tumor tissue was used for western blot and analyzed by immunochemistry. The tumor results were analyzed at the Institute of Biophysics and Informatics, Charles University in Prague, 1st Faculty of Medicine. The experiment was approved by the Commission for Experimental Animals of the medical faculty and was in accordance with the national guidelines and regulations. Statistical analyses were performed using a two-tailed unpaired Student's *t*-test.

Immunohistochemistry and immunofluorescence. Paraffin-embedded sections were obtained from the Institute of Pathology, Charles University in Prague, 1st Faculty of Medicine. Experiments were carried out with the approval of the Ethics Committee of the General University Hospital. Parallel tissue sections were stained with survivin and GLI2 primary antibodies purchased from GeneTex (Irvine, CA, USA). The detection of antigen–antibody complexes was performed using EnVision+ avidin-biotin detection system (Dako, Glostrup, Denmark). Sections were independently examined by two pathologists. Tissues were scored on a scale of 0 (negative) to 4 (highly positive) based on the intensity of staining and evaluated for statistical significance by Student's *t*-test. Only highly positive areas (scored 4), the examples of which are shown in Figure 6b, showed positive correlation in the whole sections. Thirty-five tumors of various types were examined, including non-small-cell lung carcinomas, primary melanomas, ovarian carcinomas and tubal intraepithelial carcinomas. Mice tumors were stained only for survivin. Colocalization by double immunofluorescence has been carried out on the frozen tissue sections after acetone fixation (10 min, − 80 °C). Simultaneously added primary antibodies against GLI2 and survivin were as for IHC and labeled secondary antibodies were purchased from Abcam.

Statistical analysis. Statistical significance (*P*-values) was calculated by two-tailed Student's *t*-test. S.E. values are indicated in graphs as bars in each column in the luciferase and real-time PCR assays. In the figures, values of $P < 0.05$ are marked by an asterisk, data that are not significant ($P > 0.05$) are labeled #. Values with $P < 0.01$ are not marked. Western blots were quantified by ImageJ software. In IHC and immunofuorescence sections, chosen highly positive structures were quantified and correlated by FIJI software.

Conflict of Interest

The authors declare no conflict of interest.

Author contributions

JV, KV and LO conceived the project. KV, LO, JR, JV, PD, PŽ and MZ carried out the experiments. PP contributed to and supervised the nude mice experiments. KV carried out statistical analyses. KV and LO prepared the figures and JV wrote the manuscript.

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Supplementary Information accompanies this paper on Cell Death and Disease website (http://www.nature.com/cddis)

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Figure and Table Legends to Supplementary Material

Table S1 Human cell lines used in this study were purchased from American Type Culture Collection except for marked cell lines (*): 501mel cell line was obtained from Yale University (Dr. R. Halaban), cell lines DOR, BEU and HBL are from Free University of Brussels (Dr. G. Ghanem). PA-TU-8902 cell line was obtained from Deutsche Sammlung von mikroorganismen und Zellkulturen (DSMZ), Germany. All cell lines are certified (ATCC) or authenticated in the original organization. More than half of the cell lines are primary or metastatic melanomas and small cell lung cancers. Primary melanomas are WM35 and WM1552C.

Figure S1 Increasing dose dependent effect of cyclopamine and GANT61 on the survivin promoter in SK-MEL-3 cell line shows increased survivin promoter suppression at higher doses of cyclopamine and GANT61. The cells were transfected with the survivin promoter, washed, and the agents were added for the last 24hrs of the transfection experiment.

Figure S2 **(a)** Cells (A549) were transfected increasing amounts of the indicated plasmid construct. Dual luciferase assay was performed after 48h. No or minimal increase was observed with GLI1 and GLI3 plasmids. SE values are indicated on each column. *, statistically significant ($P<0.05$), #, statistically not significant. If no mark is shown, the values are statistically significant at $P<0,01$ (all statistics is related to the control)
(b) Bordered Figure shows the comparison of the activity of GLI1 and GLI2 with NFκB and Sp1, which also activate the survivin promoter.

Figure S3 Cell lines showing minimal or no effect in survivin levels after GANT61 treatment. Cell lines in which the survivin expression was not influenced (or the changes were only infinitesimal) are depicted. Survivin was not expressed in Malme-3M and NCI-H596 cell lines. The conditions of GANT61 treatment and W. blot detection were essentially the same as in Figure 4a.

Figure S4 Linear regression depicting a weak correlation ($P<0.05$) between survivin protein abundance and survivin mRNA after GANT treatment. Quantitation of protein data were obtained from 20 μM GANT61 shown in Figure 4a with the ImageJ software. RNA levels

data were from the real time PCR experiment (Figure 4b). Only cell lines used for real time PCR are used in the statistics. The parametric Pearson correlation coefficient is shown.

Figure S5 Linear regression documenting that the survivin protein abundance and GLI protein levels do not correlate P>0.05). Quantitation of protein for survivin data was obtained from controls (0 μM GANT61) shown in Figure 4a and GLI2 from Western blots (not shown) by the ImageJ software. All 40 cell lines used were analysed. The parametric Pearson correlation coefficient is shown.

Figure S6 Survivin protein degradation. H1299 cells have increased survivin protein level after the addition of proteasome inhibitor MG132 during the GLI activity is blocked by GANT61, indicating GLI-independent protection of survivin degradation. Survivin decrease in A427 cells is justified by an RNA decrease (Figure 4b). 501mel cells react poorly to GANT61. In SK-MEL-3 cells, MG132 has not increased the survivin level at the end of GANT61 incubation, suggesting the degradation of survivin protein is protected by the activity of GLI factors. MG, MG132 (20μM); GANT, GANT61 (20μM). Cells were treated with GANT61 for 24h and MG132 was added for the last 6h of incubation. Cells were then lysed in RIPA buffer and analysed.

Figure S7 Tumor xenografts of melanoma cells. (**a,b**) Tumor apperance in controls and groups treated with GANT61 in two indicated cell lines. One of two independent experiments is shown (four mice per condition in each experiment; only 3 mice are shown because in each group one tumor did not outgrow or regressed, respectively; not shown). Similar results were obtained in both experiments. Statistical analysis was performed by a two-tailed unpaired Student's test. For 501mel cells P<0.05 values were obtained. (**c,d**) Western blots from the tumor tissue showing survivin expression. (**e**) Cell proliferation in vitro. Cell were untreated (C) or treated with 20μM GANT61 (G) for 4 days, fixed and stained. Quantification data obtained by ImageJ software are shown in each window. The growth decrease in each cell line was statistically significant (P<0.01). (**f**) Survivin expression in immunohistochemical sections of tumors from control group of mice with 501mel tumors (scored 4), SK-MEL-3 tumors (scored 3) and mice treated with GANT61 (scored 0-1). Scarcely scattered survivin positive cells were seen in tumors from GANT61 treated animals. Bar, 100 μm. (**g**) Growth curves of tumors from SK-MEL-3 cells. The results were not significant (P>0.05) due to one

tumor from the GANT61-treated group reached the final volume similar to the two control tumors. **(h)** Growth curves of tumors from inoculated 501mel cells. The tumors did not diminish, which correlated with in vitro resistance of these cells to GANT61 (correlation: $P<0.05$). Please note different Y-axis values. ▲, tumor 1; ●, tumor 2; o, tumor 3.

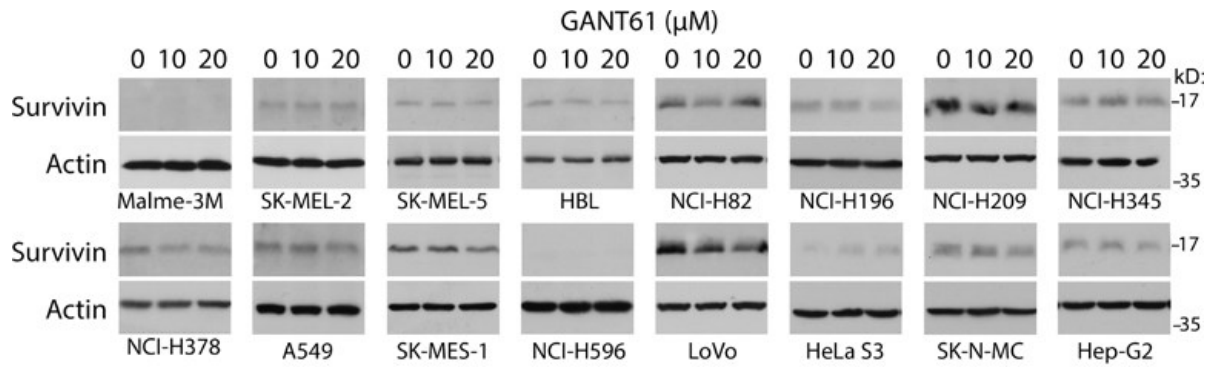| Cell line	Tumor type	Cell line	Tumor type
Malme-3M	Malignant melanoma	NCI-H378	Small cell lung cancer
MeWo	Malignant melanoma	NCI-H1299	Non-small cell lung cancer
SK-MEL-1	Malignant melanoma	A549	Non-small cell lung carcinoma
SK-MEL-2	Malignant melanoma	A-427	Non-small cell lung carcinoma
SK-MEL-3	Malignant melanoma	SK-MES-1	Squamous cell lung carcinoma
SK-MEL-5	Malignant melanoma	NCI-H596	Adenosquamous lung carcinoma
SK-MEL-28	Malignant melanoma	HCT 116	Colorectal cell carcinoma
RPMI-7951	Malignant melanoma	LoVo	Colorectal adenocarcinoma
501mel*	Malignant melanoma	SW480	Colorectal adenocarcinoma
DOR*	Malignant melanoma	C-33 A	Cervical carcinoma
BEU*	Malignant melanoma	HeLa S3	Cervical adenocarcinoma
HBL*	Malignant melanoma	SK-N-SH	Neuroblastoma
WM35	Malignant melanoma	SK-N-MC	Neuroepithelioma
WM1552C	Malignant melanoma	T98G	Glioblastoma multiforme
NCI-H69	Small cell lung cancer	MIA PaCa-2	Pancreatic carcinoma
NCI-H82	Small cell lung cancer	PA-TU-8902*	Pancreatic adenocarcinoma
NCI-H146	Small cell lung cancer	BxPC3	Pancreatic adenocarcinoma
NCI-H196	Small cell lung cancer	Jurkat, cl. E6-1	Acute T cell leukemia
NCI-H209	Small cell lung cancer	SW-13	Adrenal gland primary small cell ca.
NCI-H345	Small cell lung cancer	Hep-G2	Hepatocellular carcinoma

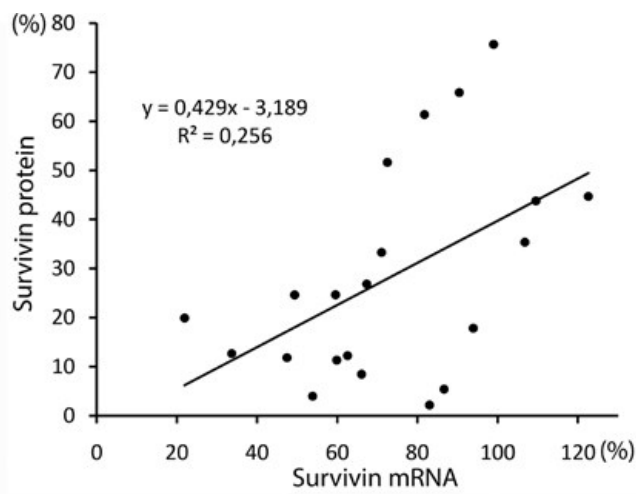**SUPPLEMENTARY TABLE S1,   Vlčková et al.**
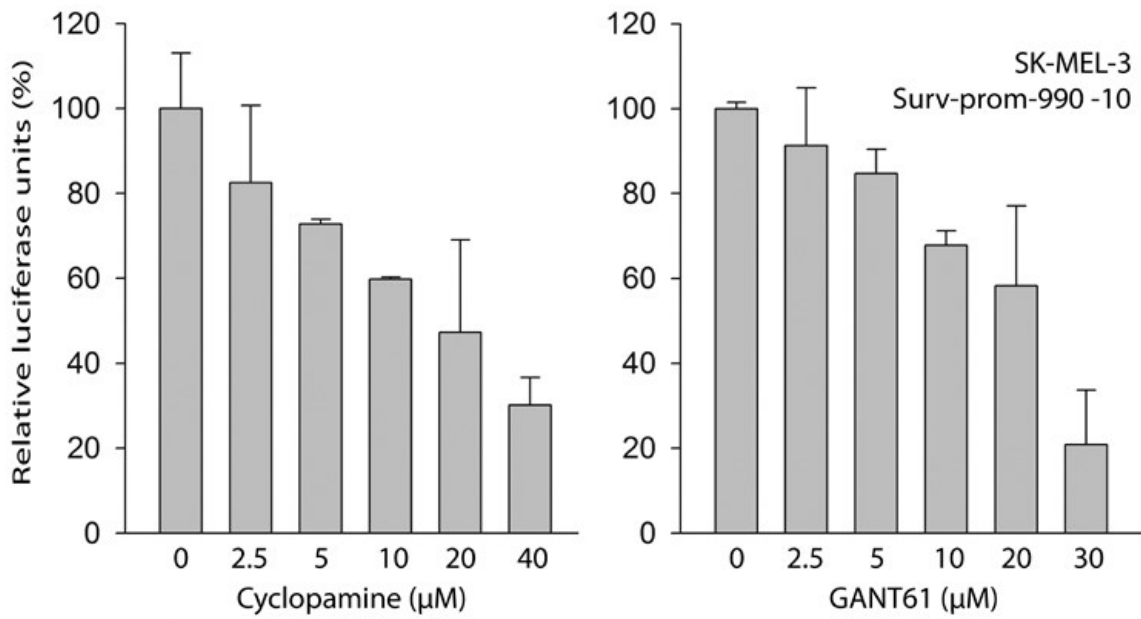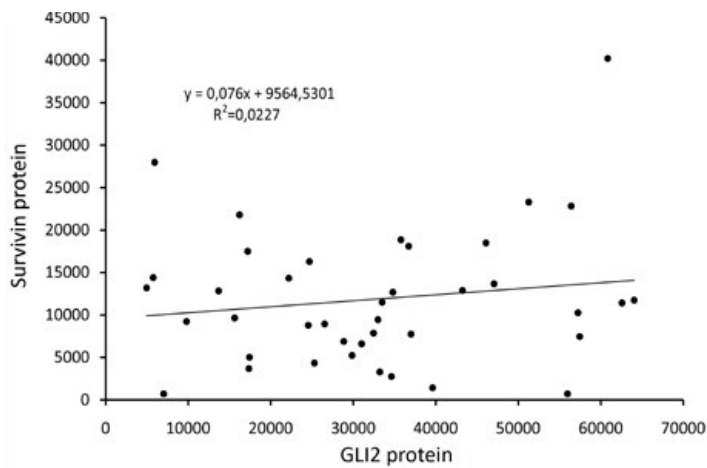
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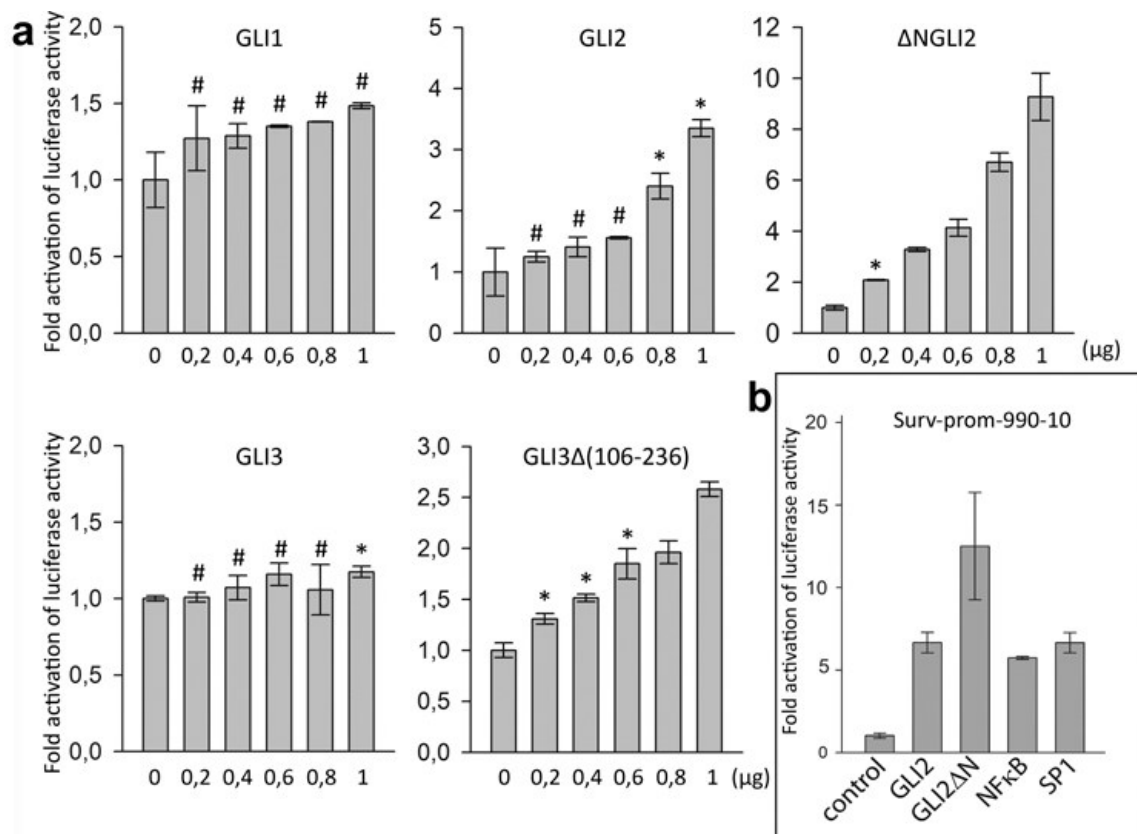
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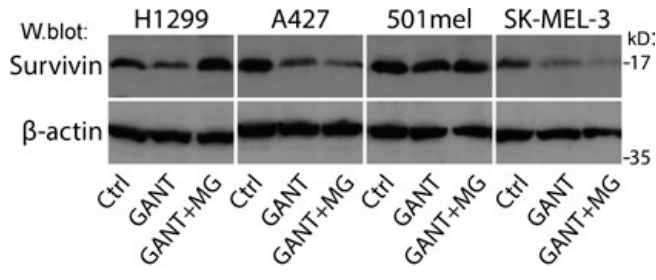
GANT61 (μM)

Supplementary Figure S3, Vlčková et al.
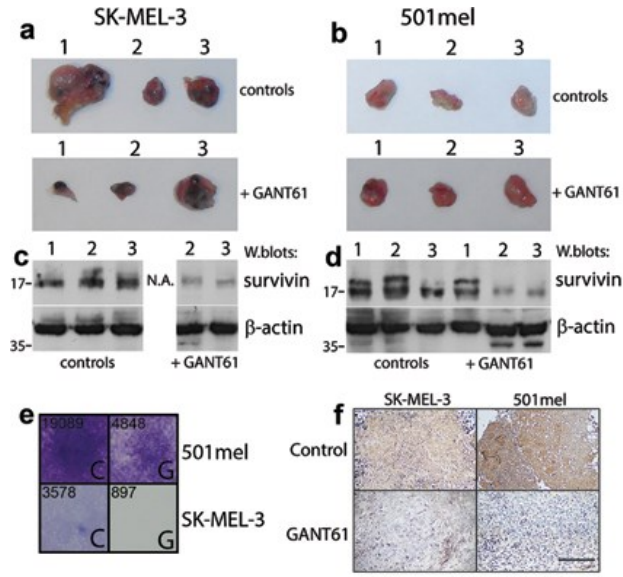

$y = 0{,}429x - 3{,}189$
$R^2 = 0{,}256$

Survivin protein

Survivin mRNA

Supplementary Figure S4, Vlčková et al.

$y = 0{,}076x + 9564{,}5301$
$R^2 = 0{,}0227$

Survivin protein

GLI2 protein

Supplementary Figure S5, Vlčková e t al.

Supplementary Figure S6, Vlčková et al.

Figure S7, Vlčková et al.
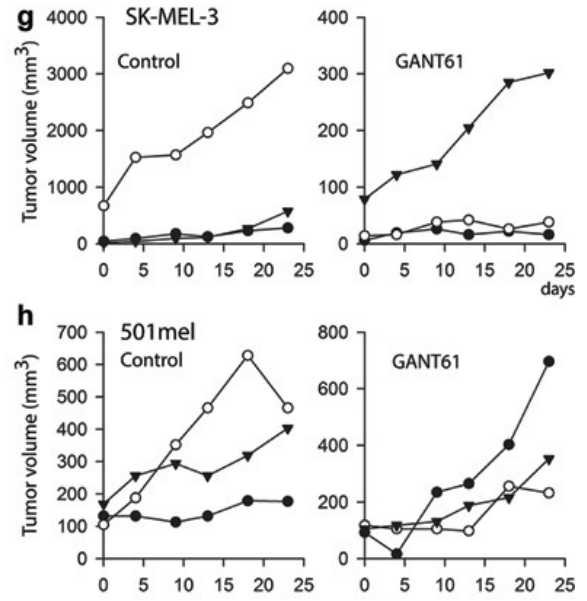
4.2 PUBLICATION II

GLI inhibitor GANT61 kills melanoma cells and acts in synergy with obatoclax

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Abstract. MEK kinase inhibitors (trametinib and selumetinib) or kinase inhibitors directed against mutated BRAF(V600E) (vemurafenib and dabrafenib) have initial encouraging effects in the treatment of melanoma but acquired resistance appears almost invariably after some months. Studies revealed mutually exclusive NRAS and BRAF activating mutations driving the MAPK/ERK pathway among human melanomas. Although combination therapy exerts significantly better antitumor cell efficacy, complete remission is rarely achieved. To employ an alternative approach, we have targeted the Hedgehog/GLI pathway, which is deregulated in melanomas, through the GLI1/2 inhibitor GANT61, alone or accompanied with the treatment by the BCL2 family inhibitor obatoclax in 9 melanoma cell lines. Thus, we targeted melanoma cells irrespective of their NRAS or BRAF mutational status. After GANT61 treatment, the cell viability was drastically diminished via apoptosis, as substantial nuclear DNA fragmentation was detected. In all tested melanoma cell lines, the combined treatment was more efficient than the application of each drug alone at the end of the cell growth with inhibitors. GANT61 was efficient also alone in most cell lines without the addition of obatoclax, which had only a limited effect when used as a single drug. In most cell lines, tumor cells were eradicated after 5-9 days of combined treatment in colony outgrowth assay. To conclude, GANT61 treatment might become a hopeful and effective anti-melanoma targeted therapy, especially when combined with the BCL2 family inhibitor obatoclax.

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Introduction

Upregulation of the Hedgehog (HH/GLI) signaling pathway is responsible for the formation and progression of a number of human cancers through the aberrant activation of transcription factors GLI (1-3). Autocrine and paracrine ligand sonic Hedgehog (Shh) binds to Patched (PTCH) receptor, thereby relieving Patched repressive activity on the 7-transmembrane protein Smoothened (SMO), which in turn causes activation of the downstream effectors, zinc finger containing GLI transcription factors (4-7). Aberrant HH/GLI pathway activity has been initially identified in basal cell carcinomas and medulloblastomas (8) and later found to be deregulated in many common human cancers such as lung tumors, pancreas, colorectal, ovarian and prostate carcinomas, glioblastomas as well as melanomas (5,7,9). Inhibitors of SMO vismodegib and cyclopamine have been used in many clinical trials. However, as the HH pathway can be upregulated non-canonically by direct activation of GLI factors by several signaling pathways (10-12), using the inhibitor of GLI activity GANT61 can overcome the possible ineffectiveness of upstream SMO inhibitors. GANT61 prevents the binding of GLIs to DNA (13) while fully preserving their expression.

GLI2 has been shown to control the invasiveness and metastatic potential and to contribute to the epithelial-to-mesenchymal transition in melanoma (14). Melanomas express MITF (microphthalmia-associated transcription factor), a crucial factor in the pigment cell transcriptional circuitry, activating a large number of genes with various functions (15-17). GLI2 expression was reported to be inversely correlated with MITF expression. Thus, high GLI2 and low MITF levels characterize the invasive cell phenotype in melanoma (18,19). High GLI2 expression in melanoma was achieved through the HH and TGF-β/SMAD pathways (20,21). HH/GLI1 signaling has an essential role in controlling self-renewal and tumor initiation of melanoma and GANT61 has been reported to reduce the number of melanomaspheres formed from cells with features of tumor initiating stem cells (22). However, despite the importance of HH/GLI pathway and GLI2 in the melanoma development, the data that would determine the effect of GANT61 on melanoma cell cultures *in vitro* is lacking. The treatment of melanoma by inhibitors against BRAF(V600E)

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or MAPK pathway were initially promising, but resistance appeared almost invariably after months through multiple mechanisms (23,24). Other treatment approaches are therefore needed in melanoma.

In the present study, we undertook treatment of 9 melanoma cell lines with GANT61, a downstream Hedgehog/GLI pathway inhibitor, and performed a combined incubation of GANT61 with obatoclax, a BCL2 family inhibitor. We identify that melanoma cells are efficiently eliminated by GANT61 through apoptosis. GANT61 with obatoclax reveal synthetic lethality in cell lines *in vitro* as assessed by colony formation assays. Although the results require the *in vivo* verification, this targeted combined therapy is promising to be beneficial for melanoma patients irrespective of the BRAF or NRAS mutational status.

Materials and methods

Promoters and reporter assays. The ΔNGLI2 expression vector was previously described (25). The MITF promoter-reporter construct has been described (26). Professor Fritz Aberger (University of Salzburg) provided the Patched promoter plasmid and Professor Rune Toftgard (Karolinska Institutet) the 12xGLI reporter plasmid. For reporter assays, melanoma cells were seeded in 12-well plates and transfected at 70-80% confluency in fresh medium with LipoJet (SignaGen Laboratories, Rockville, MD, USA) according to the instructions of the manufacturer. Dual-luciferase assay kit (Promega, Madison, WI, USA) was used to determine promoter-reporter activity.

Cell lines. Melanoma cell lines MeWo, SK-MEL-3, SK-MEL-5, SK-MEL-28, WM35, WM1552C and SW13 line (human cervical carcinoma) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Beu and Hbl cells were previously described (25). 501mel cell line was obtained from Dr Ruth Halaban (Yale University) and maintained in RPMI-1640 medium. Hbl and SW13 were grown in Dulbecco's modified Eagle's medium (DMEM) and all other cell lines were cultivated in EMEM medium. All types of media (Sigma-Aldrich, St. Louis, MI, USA) were supplemented with 10% fetal calf serum (FCS; Life Technologies, Carlsbad, CA, USA), L-glutamine and antibiotics (Sigma-Aldrich).

Cell viability assay. Cells (50,000/well) were seeded in 12-well plates (Nunc, Roskilde, Denmark) and treated the next day with 25 μM GANT61 (Selleckchem, Munich, Germany) or DMSO (Sigma-Aldrich) (control) for 72 h. Cultivation medium was removed and cell viability was determined with MTT cell viability assay kit (Sigma-Aldrich). Experiments were performed in duplicate and data are expressed as a mean of duplicate measurements (mean ± SD) in percentage of control-treated cells (100%). Two independent experiments were carried out with similar results and one experiment is shown.

Cell proliferation assay. To perform colony outgrowth assay, subconfluent cells were trypsinised and seeded in 12-well plates (day 0). The next day, cell lines were treated with inhibitors at concentrations of 20 μM GANT61 or 100 nM obatoclax (Selleckchem), or their combination for the indicated time

intervals (9 days maximum). The plates were fixed in 3% paraformaldehyde solution in PBS and stained with 1% crystal violet. Two experiments were performed in duplicate. Results of both experiments were similar. The density of the remaining stained cells was quantified with the ImageJ software and the results of one experiment are shown.

Soft agar assay. Assay was performed in 60-mm dishes containing 0.6% lower layer of Noble agar (Difco, Radnor, PA, USA). Cells (5x10⁴) were seeded in the upper agar (0.4% agar in cultivation medium containing 15% FCS), overlayed with the cultivation medium with 15% FCS and DMSO or inhibitors. The media with inhibitors or control vehicle were refreshed twice a week. After 21 days colonies were stained with p-indonitrotetrazolium violet (Sigma-Aldrich), counted, photo-documented and quantified by the ImageJ software.

Detection of apoptotic cells. DNA fragmentation as a hallmark of advanced apoptosis was detected by flow cytometric and microscopic analyses. SK-MEL-3 and SK-MEL-5 cells were treated with 20 μM GANT61 or DMSO (control) for 48 h and then trypsinized, combined with detached cells, washed with PBS, incubated in the DNA extraction buffer (192 ml of 0.2 M Na_2HPO_4 with 8 ml of 0.1 M citric acid, pH 7.8) for 10 min at room temperature, washed again with cold PBS, resuspended in PBS containing 5% BSA (Merck, Darmstadt, Germany) and stained with propidium iodide (Sigma-Aldrich) (20 μl of 0.5 mg/ml solution per 1 ml of cell suspension). RNAse (Sigma-Aldrich) was added simultaneously (2 μl of the 20 mg/ml stock per 1 ml of cell suspension). Cell histograms were acquired on a FACSCanto flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) with the FACSDiva VI acquisition and analysis software. To obtain microscopic images of apoptotic nuclei, cells were grown in 2-well chambers (NUNC) and treated with GANT61 as above. The remaining attached cells were then fixed with 3% paraformaldehyde in PBS. Slides were coverslipped with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) to visualize the nuclei. Images were acquired on an Olympus BX61 microscope (Olympus, Tokyo, Japan).

Western blot analysis. Cell extracts for immunoblotting analysis were prepared by lysis the cells in RIPA buffer (1% NP-40, 150 mM NaCl, 5 mM EDTA, 0.5% sodium deoxycholate, 50 mM Tris-HCl pH 7.5, 0.1% SDS) with added protease and phosphatase inhibitors: 1 μg/ml of leupeptin, aprotinin and pepstatin, and cOmplete and PhosStop (Roche Diagnostics, Mannheim, Germany) as recommended by the manufacturer. Protein extracts (30 μg) in sample loading buffer (50 mM Tris-HCl pH 6.8; 2% sodium dodecyl sulphate; 100 mM dithiotreitol; 10% glycerol; 0.1% bromophenol blue) were heated to 98˚C for 2 min and proteins were separated on 10% polyacrylamide gels. After transfer onto the PVDF membrane (Millipore, Billerica, MA, USA), the membranes were blocked in 5% Blotto (Santa Cruz Biotechnology, Dallas, TX, USA) in PBS containing 0.1% Tween-20 (Sigma-Aldrich) at room temperature for 1 h. The incubation with the primary antibodies against GLI2 (GTX46056), purchased from GeneTex (Irvine, CA, USA), MITF (Lab Vision, Fremont, CA, USA), or β-actin (AC-74) from Sigma-Aldrich was conducted
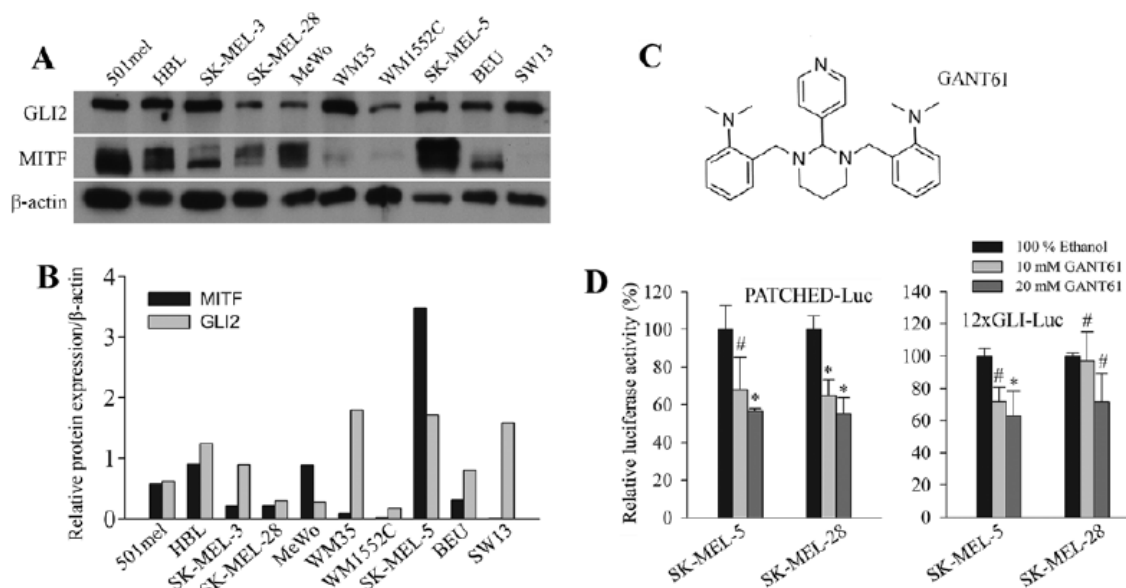
Figure 1. Expression of GLI2 and MITF in melanoma cell lines and inhibition of HH/GLI responsive promoters by GANT61. (A) Nine melanoma cell lines and a non-melanoma SW13 line were tested by western blotting with anti-GLI2 or anti-MITF antibody and control anti-β-actin antibody. (B) Quantification of western blot results from (A) expressed as relative expression of GLI2 or MITF corrected to the β-actin expression. Control cell line SW13 does not express MITF. (C) Chemical structure of GANT61. (D) Inhibition (%) of HH/GLI-activated promoters by GANT61. Cells were transiently transfected with the promoter-luciferase plasmids Patched or 12xGLI (0.3 μg) together with the expression plasmid ΔNGLI2 (0.7 μg) to increase the promoter activity. Cells were treated with GANT61 for 20 h before harvesting. Control value was set as 100% (luciferase arbitrary units). #Indicates statistically not significant; *P<0.05, statistically significant.

for several hours at room temperature, diluted 1:1,000 in the blocking solution. After washing in PBS-0.1% Tween, 1 h incubation in the HRP-labelled secondary antibody (Cell Signaling Technology, Danvers, MA, USA) and washing, signals were detected by the Pierce ECL chemiluminiscent detection reagent (Thermo Fisher Scientific, Waltham, MA, USA). In western blot experiments determining the effect of GANT61 on MITF protein levels, GANT61 was left on cells for 20 h and the cells were harvested (Fig. 2).

Statistical analysis. Statistical comparison was carried out using the unpaired Student's t-test. Significance was set at P<0.05. Standard error (SE) is shown for triplicate or duplicate samples in promoter-reporter or cell viability assays, respectively. The combination index (CI) was calculated using CalcuSyn software. GLI2 and MITF proteins levels were corrected after densitometry (using AIDA image analyzer software) to western blots with the β-actin (control) expression (Figs. 1B and 2B).

Results

Expression of GLI2 in melanoma cell lines. GLI2 was shown to be an essential protein for maintaining the pro-oncogenic phenotype in melanoma (14). GLI2 expression was determined in melanoma cell lines and control non-melanoma cells SW13 (adrenal gland carcinoma) by western blotting. The BRAF and NRAS mutational status of melanoma cell lines used is provided in Table I (27-32). GLI2 was present in all cells examined (Fig. 1A). Expression was noted also in the 501mel cells, which were previously reported to be GLI2 negative (18).

In our recent study, these cells were also clearly GLI2 positive for both protein and mRNA, as detected by real-time PCR (25) and RT-PCR (data not shown). While the use of a different antibody might explain the difference in the western blot result, we can not clarify the discrepancy in the result of RNA level in 501mel cells at present. The western blot band density was quantitated and corrected to β-actin levels (Fig. 1B). In 6 of 9 cell lines, similar trend of inverse correlation between GLI2 and MITF was observed, as that previously reported (18). We observed this trend in different cell lines (with the exception of 501mel) than those employed by others (18). Since we have detected GLI2, a protein implicated in melanoma invasion and metastasis, in all cell lines in our panel, they are predicted to be good models for exploring the effect of GANT61. The chemical structure of GANT61 is shown in Fig. 1C.

GANT61 inhibits GLI2-dependent promoter reporters. To test whether GANT61 inhibits GLI-dependent transcription, we examined the activity of known HH/GLI responsive promoter reporters, the 12xGLI arteficial super-promoter and the Patched promoter. After stimulation of these promoters by cotransfecting ΔNGLI2, the most effective GLI construct which was also the most effective stimulant for the newly discovered GLI2 target survivin promoter (25), increasing concentrations of GANT61 were added for 20 h. The activity of both promoters was evidently decreased in two melanoma cell lines tested (Fig. 1D), albeit most results at 10 μM concentration and one result at 20 μM of GANT61 (the 12xGLI promoter in SK-MEL-28 cells) were not statistically significant (due to high SE values). Considering this observation, together with the finding of the uniform presence of GLI2 levels in mela-

Table I. Mutational status of BRAF and NRAS in melanoma cell lines.

| Cell line	BRAF	NRAS	Author/Ref.
501mel	V600E	wt	Packer et al (27)
Hbl	wt	wt	Herraiz et al (28)
SK-MEL-3	V600E	wt	Hao et al (29)
SK-Mel-28	V600E homozyg.	wt	Smalley et al (30)
			Singh et al (31)
MeWo	wt	wt	Domenzain-Reyna et al (32)
WM35	V600E	wt	Smalley et al (30)
WM1552C	V600E	wt	https://www.wistar.org/lab/meenhard-herlyn-dvm-dsc/page/melanoma-cell-lines-0
SK-MEL-5	V600E homozyg.	wt	Singh et al (31)
			http://www.sanger.ac.uk/genetics/CGP/CellLines/
Beu	Not known	Not known	
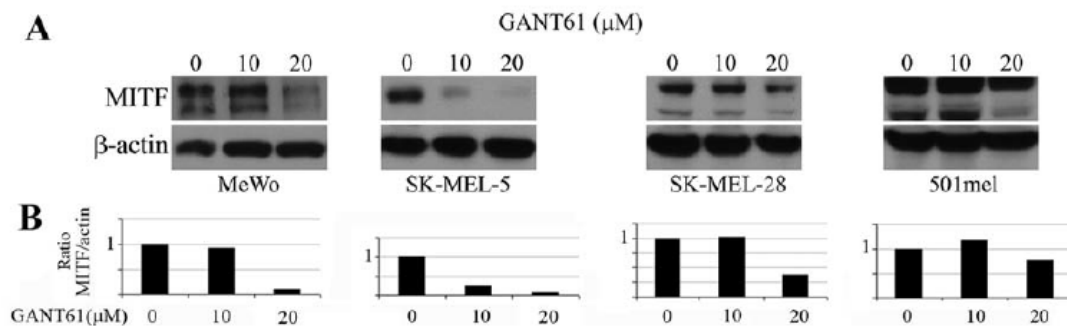
Figure 2. GANT61 treatment results in decrease of endogenous MITF protein level in melanoma cells. (A) Western blot analysis of cells incubated in medium with 0, 10 and 20 μM GANT61 for 20 h, harvested, and protein lysates were analysed with the anti-MITF and control β-actin antibodies. (B) Quantification of western blots as relative expression of MITF compared to the β-actin expression.

noma cells (Fig. 1A), we hypothesized that GANT61 might possibly affect the growth of melanoma cells.

GANT61 decreases the MITF expression, cell viability and outgrowth of melanoma cells in soft agar. Since GLI2 expression was reported to be inversely correlated with the MITF expression (18) and GLI2 was described to repress MITF transcription (19), we expected that GANT61, a GLI1/2 inhibitor, would increase the MITF levels in melanoma cells. However, we observed no effect in MITF protein levels on western blots after treatment of cells with 10 μM GANT61 (Fig. 2A) in 3 melanoma cell lines and a massive inhibition of MITF level in SK-MEL-5 cells even at 10 μM (see Discussion) (Fig. 2A). The 20 μM concentration inhibited MITF levels strongly in 3 cell lines and only negligibly in 501mel cells. These results are clearly evident after quantitation and correction of the MITF levels to β-actin levels (Fig. 2B).

We next addressed whether GANT61 has effects on cellular phenotype of melanoma cells. The viability of our panel of 9 melanoma cell lines and 3 non-melanoma cell lines was drastically diminished after 72-h incubation with 25 μM GANT61. Only 5-35% of viable cells remained. Only one of controls (A549 cells, lung carcinoma) was resistant as viability decreased by <10% (Fig. 3A). The soft agar growth

of the two most sensitive lines based on the viability assay, SK-MEL-3 and Beu, was examined. Whereas colony formation of SK-MEL-3 cells was completely abrogated by 20 μM GANT61 after three weeks, Beu cells formed only small number of colonies (related to control) after quantitation of the dish images (Fig. 3D). We tested also 100 nM obatoclax and its combination with GANT61 for clonogenic growth. Obatoclax completely abrogated soft agar colony outgrowth in SK-MEL-3 cells and it was more effective than GANT61 in Beu cells. Combined treatment completely prevented the colony formation in both cell lines (Fig. 3D), suggesting a synergistic effect in eradication of melanoma cells.

GANT61 induces massive apoptosis in melanoma cells. It is well established that GANT61 causes apoptosis in cancer cells (5,23-35). The possible accompanying caspase and PARP cleavage can be dependent on the cell type. To demonstrate whether GANT61 eliminates viability of melanoma cells through apoptosis, flow cytometric analysis was performed after 2 days of cultivation in at concentration of 20 μM. Massive sub-G1 phase indicating the DNA fragmentation was observed in SK-MEL-3 and SK-MEL-5 cells (Fig. 3B). Together with examining the remaining attached cells, we analysed also the detached cells, possibly
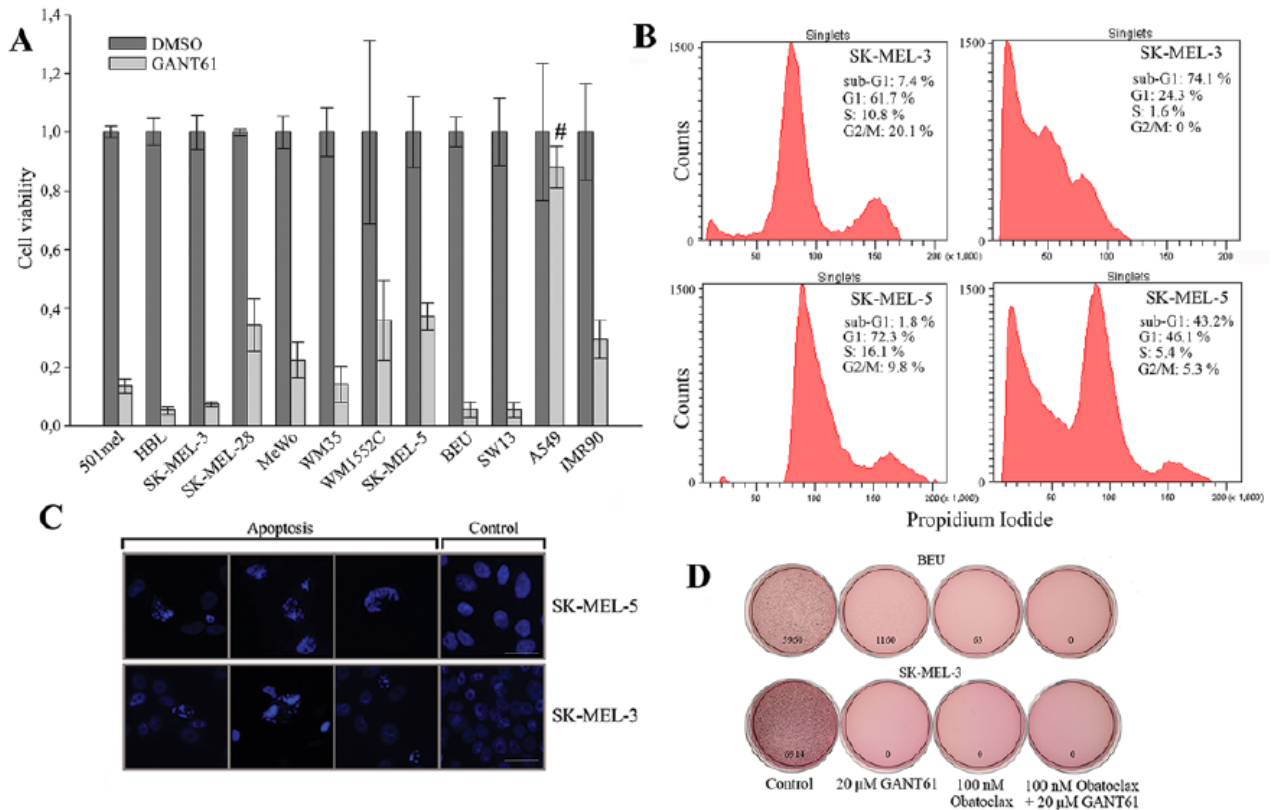
Figure 3. Decrease of viability, colony outgrowth in soft agar, and induction of apoptosis by GANT61. (A) Viability was tested on 9 melanoma cell lines and 3 control non-melanoma cell lines (SW13, A549 and IMR90) as indicated. After 3 days with GANT61 at 25 μM, cell viability was estimated by the MTT assay. (B) Two melanoma cell lines (SK-MEL-3 and SK-MEL-5) were treated with 20 μM GANT61 for 48 h. Apoptosis was measured by the propidium iodide method using flow cytometry. Both detached and remaining attached cells were measured. Cell cycle phases are indicated as inserts where sub-G1 phase represents the percentage of apoptotic cells. Left, control cells; right, cells treated with GANT61. (C) Apoptotic nuclei stained by DAPI. Three examples of apoptosis and one control are shown for each cell line. (D) Soft agar assay performed with two melanoma cell lines. The assay was carried out as described in Materials and methods and results of quantitation are depicted at the bottom of each dish.

explaining the appearance of such profound apoptosis. The high sensitivity of SK-MEL-3 correlated with the colony formation assays in which these cells appeared to be the most susceptible to GANT61 treatment (Fig. 4). To further substantiate that apoptosis was the mechanism of killing the melanoma cells, the same GANT61 treatment as used for flow cytometry was applied to cell monolayers and the remaining attached cells were mounted in DAPI-containing medium. Nuclei fragmentation and chromatin condensation indicating apoptosis were clearly seen, whereas normal nuclei were observed in control cells (Fig. 3C). Taken together these findings indicate that GANT61 induces powerful apoptosis in melanoma cells.

Obatoclax in synergy with GANT61 efficiently eradicate melanoma cells in vitro. Having shown that GANT61 can effectively abolish the survival of melanoma cells, we decided to investigate the possible synergistic effect of GANT61 when used with inhibitors related to melanoma treatment. Firstly, we studied whether selumetinib, a MEK inhibitor, and AZD5363, an AKT kinase inhibitor, could improve the efficacy of 20 μM GANT61 in melanoma cells. We used 300 nM concentration of both drugs, which

is sufficiently high concentration considering that the IC_{50} values for both selumetinib and AZD5363 targets (MEK and ERK1/1 or AKT, respectively) are below 20 nM (www.selleckchem.com). The experiments were designed similarly as shown in Fig. 4. However, the combined incubation of cell lines in the panel did not lead to improvement of the use of GANT61 alone (data not shown). Expectedly, selumetinib or AZD5363 alone (at 300 nM concentration) did not reveal any considerable effect on any cell line even after 9 days of treatment (results not shown). We therefore used obatoclax, an inhibitor of the BCL2 family of anti-apoptotic proteins. Although it showed no effect on any cell line when tested alone (at 100 nM concentration) (Fig. 4), obatoclax accelerated the effect of GANT61 treatment in 6 of 9 melanoma cell lines (501mel, Hbl, SK-MEL-28, MeWo, WM1552C and SK-MEL-5), whereas no additional effect was observed in the remaining 3 cell lines (SK-MEL-3, WM35 and Beu). In SK-MEL-3 (the most GANT61 sensitive melanoma cells), Beu melanoma cells and SW13 non-melanoma control cells, the cell death appeared even earlier when GANT61 was applied alone (Fig. 4). Combination index (CI) was calculated (Table II) revealing the 'antagonistic' effect in the Beu and SK-MEL-3 cell line, evidently due to the earlier effect
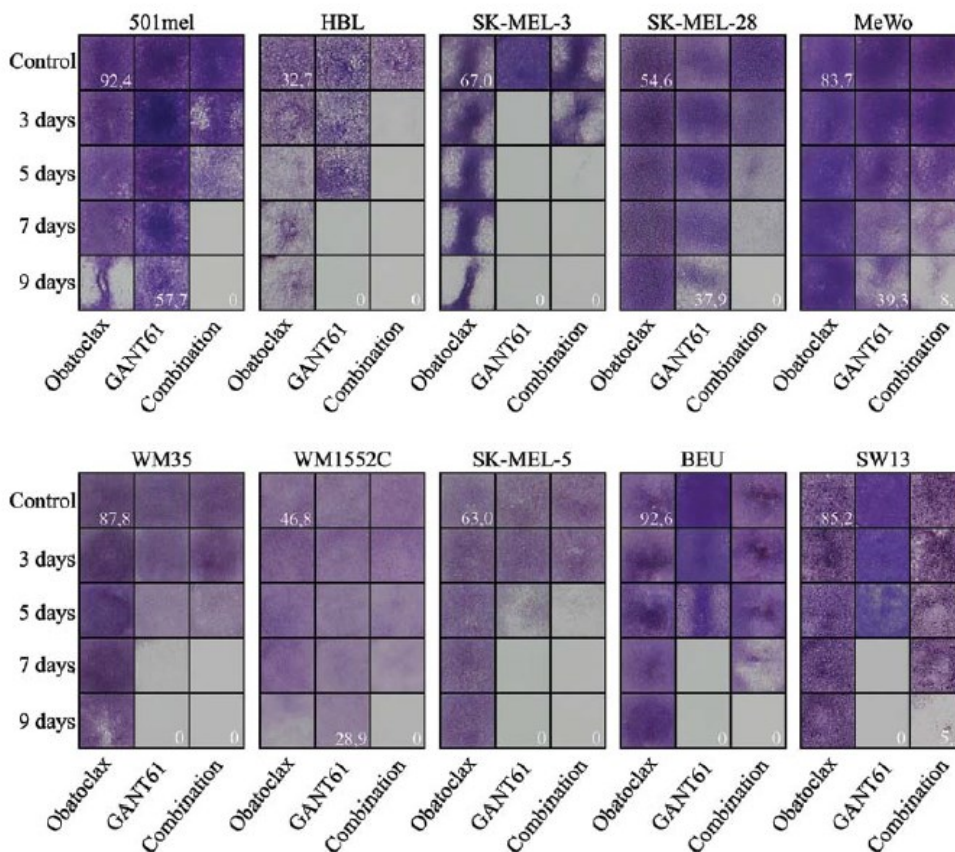
Figure 4. Proliferation of nine melanoma cell lines and SW13 cells in the presence of GANT61 (20 μM), obatoclax (100 nM), or their combination. Colony formation assay was monitored for 9 days. Plates were then fixed, washed and stained with crystal violet. All wells were performed in duplicate and only one well is shown. The values between duplicates did not deviate by >5%. The values for day 1 (control) and day 9 (GANT61 and obatocalax) are indicated. Combination index has been calculated for each cell line and its values are indicated in Table II.

Table II. Combination index (CI) of the analyzed melanoma cell lines.[a]

Cell line	Combination index (CI)
501mel	0.091
HBL	0.14
SK-MEL-3	1.798[b]
SK-MEL-28	0.121
MeWo	0.414
WM35	0.95
WM1552C	0.812
SK-MEL-5	0.216
BEU	1.88[b]
SW13 (non-melanoma)	10.949

[a]CI values between 0.9 and 1.1 indicate an additive effect; values between 0.7 and 0.9 are a moderate synergism; values <0.7 represent a strong synergism. Antagonism is represented by CI values >1.1. [b]Please see the text for details.

of GANT61 alone than the combined treatment. Additive CI was calculated for WM35 cell line (Table II). Nonetheless, at the end of the experiment (day 9), all 9 melanoma cell lines revealed the best effect of GANT61 + obatoclax combination (the bottom right field in each block; Fig. 4).

Thus, GANT61 efficiently eliminated melanoma cells in the clonogenic growth assay and exhibited synthetically lethal effect with obatoclax in most melanoma cell lines.

Discussion

Malignant melanoma continues to increase in incidence worldwide. The treatment of advanced melanoma with low molecular weight inhibitors directed to mutated BRAF or MAPK inhibitors results in acquired resistance. It has been found that some kinase inhibitors induce a specific secretome increasing the tumor outgrowth and metastasis, supporting growth of cell clones with drug-resistance, actually promoting tumor progression after treatment (24). Although the combination of drugs improved the therapy, it remains questionable if MAPK signaling pathway, despite its deregulation virtually in all melanomas, is an ideal target for the therapy. More mechanisms are involved in the acquired resistance to the MAPK signaling in melanoma (23,36-39). Therefore, alternative approaches should also be considered.

GLI2 transcription factor has been recognized as important pro-invasive factor through its contribution to maintaining the

cancer stem cell subpopulations and progression to metastasis in many cancers (21,40-43). Many pro-oncogenic targets for GLI2 has been identified. Recently, we found that survivin is also a GLI2 target in about half of a large panel of human tumor cell lines, with several tumor lines being absolutely reliant on GLI2 for survivin epression (25). In keeping with this, both survivin expression and Hedgehog signaling are active during embryonic development and in tumor cells, while normal adult non-proliferating cells are silent or display very low activities of both HH/GLI and survivin expression. Thus, HH/GLI can widely contribute to the antiapoptotic activity through GLI2-directed survivin expression in cancer cells. GANT61 is a powerful and specific inhibitor of GLI1/2 factors activity. It also progresses into the clinic as a promising anticancer drug for many types of tumors. We therefore reasoned that it could constitute a possible effective agent in melanoma. We have shown here that it causes apoptosis in melanoma cells and inhibits their survival as assessed by colony formation assay. As the emerging data from both preclinical and clinical studies suggest that resistance is likely to occur following the monotherapy, we have successfully treated melanoma cells with GANT61 in combination with the BCL2 inhibitor obatoclax. Deregulated expression of BCL2 family proteins is known to play a central role in the resistance of melanoma to apoptosis (39).

In the present study, obatoclax accelerated the eradication of tumor cells in most melanoma lines (6 of 9), whereas we have not observed its toxicity against any melanoma cell line when used alone at 100 nM concentration. In contrast, we found that either MEK inhibitor selumetinib or AKT inhibitor AZD-5363 did not synergize with GANT61 in eradication of melanoma cells. As obatoclax is effective towards the whole BLC2 family, the proposed enhancement of apoptosis induced by obatoclax may improve the results obtained with GANT61 and prevent the development of resistance in patients. To conclude, the presented combined treatment could be the basis for further research based on melanoma cell elimination through apoptosis and independently of BRAF or NRAS mutations and the activity of MAPK signaling.

Concomitantly, we estimated here the level of a pivotal transcription factor and melanoma oncogene MITF after GANT61 treatment. GLI2 has been demonstrated previously to repress transcription of MITF (19) and reciprocal expression of GLI2 and MITF is involved in the 'phenotype switching' model where high GLI2 functions to promote melanoma cell phenotypic plasticity and invasive behavior (18). Surprisingly, we found that the blockade of GLI2 activity by the addition of GANT61 does not increase the MITF protein, but rather has no effect or causes a decrease of its level. We therefore hypothesize that GLI2 may not be the MITF transcriptional repressor in every cell context, or GLI2 activity can eventually block the proteasomal degradation of the MITF protein by a yet unknown mechanism, similarly as observed for survivin in some tumor cell lines (25). However, although it is difficult to interpret these data at present, the findings together argue that in a specific cell context (SK-MEL-5 cells), GLIs are important to maintain the MITF cellular level. We speculate that the MITF level might not be a causative factor in the melanoma phenotype switching, but rather is a consequence that mirrors the phenotype changes governed by other factors. GLI2, on the other hand, activate genes that confer the invasive phenotype to melanoma cells and together with other factors such as SOX2, ZEB1, TWIST or SNAIL1 (44-46) could contribute to stem cell-like and metastasis prone properties of small subpopulations of cells in melanoma.

Acknowledgements

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4.3 PUBLICATION III

Article

Widespread Expression of Hedgehog Pathway Components in a Large Panel of Human Tumor Cells and Inhibition of Tumor Growth by GANT61: Implications for Cancer Therapy

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Abstract: The sonic Hedgehog/GLI signaling pathway (HH) is critical for maintaining tissue polarity in development and contributes to tumor stemness. Transcription factors GLI1–3 are the downstream effectors of HH and activate oncogenic targets. To explore the completeness of the expression of HH components in tumor cells, we performed a screen for all HH proteins in a wide spectrum of 56 tumor cell lines of various origin using Western blot analysis. Generally, all HH proteins were expressed. Important factors GLI1 and GLI2 were always expressed, only exceptionally one of them was lowered, suggesting the functionality of HH in all tumors tested. We determined the effect of a GLI inhibitor GANT61 on proliferation in 16 chosen cell lines. More than half of tumor cells were sensitive to GANT61 to various extents. GANT61 killed the sensitive cells through apoptosis. The inhibition of reporter activity containing 12xGLI consensus sites by GANT61 and cyclopamine roughly correlated with cell proliferation influenced by GANT61. Our results recognize the sensitivity of tumor cell types to GANT61 in cell culture and support a critical role for GLI factors in tumor progression through restraining apoptosis. The use of GANT61 in combined targeted therapy of sensitive tumors, such as melanomas, seems to be immensely helpful.

Keywords: Hedgehog; GLI; tumor cell lines; GANT61; apoptosis

1. Introduction

The Hedgehog (HH) signaling pathway is a morphogenesis pathway crucial for the growth and patterning of various tissues during embryonic development [1,2]. The morphogen sonic Hedgehog binds the transmembrane receptor Patched (PTCH), which activates another transmembrane protein Smoothened (SMO) and triggers the HH pathway that influences the expression of many genes through the activation of transcription factors GLI1 and GLI2. GLI3 activates only exceptionally and behaves rather as a suppressor. HH components are highly conserved from fly to human [3]. Initially, the HH pathway was linked to the etiology of basal cell carcinoma and medulloblastoma [4–8]. The pathway transcriptionally upregulates the expression of survivin in more than half of analyzed cell lines [9]. Accumulating evidence suggests that the HH pathway is critical for almost all tumors. It has been found that HH signaling plays key roles in formation and maintenance of cancer stem cells (CSC), tumor stemness, and acquisition of epithelial-to-mesenchymal transition (EMT) in tumors. Since EMT

is important and responsible for cancer cell invasion, metastasis, drug resistance, and tumor recurrence, the HH signaling pathway is now believed to be an important target for cancer therapy [10–13]. The HH pathway and GLI factors thus appear to be promising targets for cancer therapy [14]. Several cancers were shown to be sensitive to HH inhibition, such as lung cancer (both non-small cell lung cancer (NSCLC) [15–18] and small cell lung cancer (SCLC) [19,20]). Many reports highlight the importance of the HH pathway in pancreatic cancer and the usefulness of its inhibition [21–24]. The HH pathway was described to be crucial for the pancreatic cancer development and HH inhibition caused autophagy in CFPAC-1 cells in vivo and in mouse xenografts [25]. GLI1 promoted EMT and metastasis in pancreatic cells in a genome-wide screening study [26]. In many other cancer types, the HH pathway inhibition decreases the oncogenicity and has been beneficial for the patients. Melanomas critically require HH signaling [27–29], presumably with activated RAS-MAPK and AKT signaling cascades [27]. HH has been described to promote oncogenesis in leukemias [30–34], bladder cancer [35], and prostate cancer [36–39].

Global significance of the HH pathway for tumor initiation, progression, and metastasis is documented by additional literature. Mounting evidence indicates that HH signaling is required for the maintenance of glioblastoma and its CSC population [40,41]. GLI2 has been identified as a target for the treatment of osteosarcoma [42] and the HH pathway has been reported to be important for osteosarcoma progression and metastasis [43]. HH signaling produces self-renewal in embryonal rhabdomyosarcoma [44], has a critical role in the growth of neuroblastoma [45], ovarian cancer [46,47], hepatocellular carcinoma [48], colon carcinoma [49,50], and is pivotal for forming breast cancer CSC [51] and bone metastases [52]. Rhabdoid tumors and cell lines lack INI1 (SMARCB1/SNF5) tumor suppressor. This is a causative event in these tumors. This protein is central in the nucleosome remodeling complex SWI/SNF and is also rarely absent in rhabdomyosarcomas. It was found that INI1 binds GLI1. In the presence of INI1, the HH pathway is silent and the loss of INI1 triggers the activation of the HH pathway in rhabdoid tumors [53]. Ectopic INI1 is able to rescue the nonmalignant phenotype in rhabdoid tumor cell lines. This implies that an activated HH cascade causes this tumor type. This is intriguing because INI1 is present in all other cells including tumor cells with an elevated HH pathway activity (above). This implies a very specific cell context in rhabdoid tumors and suggests the HH pathway as a target for their treatment.

Several studies have implicated a noncanonical activation of the HH route in tumors, thus abrogating the necessity of upstream ligand signaling. Through this mechanism, GLI factors can be activated directly by many different mechanisms upregulated in tumor cells, predominantly operating in RAS/MAPK, Wnt, or AKT pathways [38,54–57]. As an example, KRAS activates GLI1 in pancreatic cancer cells [58], an androgen receptor (AR) protects GLI3 from proteolytic cleavage [38], and HH can be activated by the mTOR/S6K1 signaling [59]. This allows the processing of the deregulated HH pathway without the membrane signaling through direct aberrant GLI factors stimulation with the consequent expression of their prooncogenic targets. Here, we present results showing that the main components of the HH pathway are invariably expressed across a large panel of tumor cells of various cancer types. The most potent HH inhibitor GANT61 suppressed proliferation more or less in about half of tumor cell lines (the sensitive cells were eradicated presumably through apoptosis) and is a prime candidate as a compound for the combined therapy in many tumor types.

2. Results

2.1. Broad Expression of HH Cascade Components in Human Tumor Cell Lines

We were interested in studying whether constituents of the HH pathway are invariably present in several tumor cell types or if some components are missing. It would potentially disable the activation of HH pathway in human cancer cell lines. A large screen has been performed and Western blots have shown complete expression of the main HH components in all tumor cells (Figure 1). Noteworthy, two lines expressed negligible GLI1 (G-401 and NCI H446), whereas GLI2 in them was

expressed abundantly. In some other cells, GLI2 was low but GLI1 sufficiently expressed (RPMI-7951, Calu-1, HeLa S3, H-209, H-345, and Jurkat). The SuFu level was low in Hbl and H69 cells. In some tumor cell lines, expression of GLI3 was lower (DOR, Saos-2, and H-196). GLI3 is nevertheless only exceptionally necessary for processing of HH signals, whereas either GLI1 or GLI2 are generally required. Patched was weak in Saos-2 and Jurkat cells, and SMO was weakly expressed only in H-69 cells.
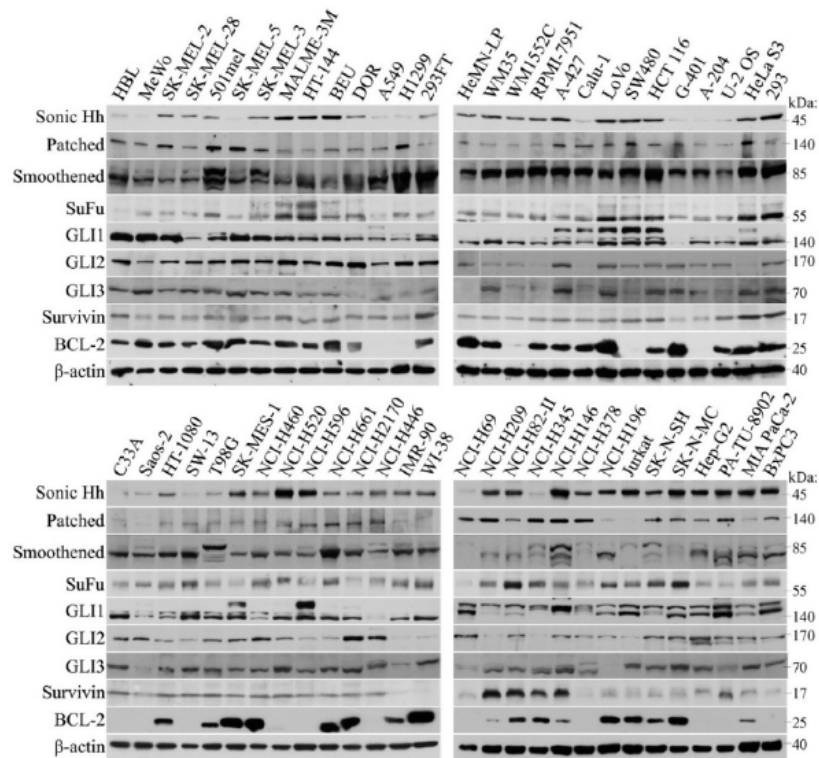

Figure 1. Panel of protein expression pattern of HH signaling components. Western blots made in RIPA extracts (30 μg) were probed with indicated antibodies. With some small exceptions, all HH proteins were expressed, although sometimes the expression level was weaker (see text). Survivin, an HH target, was invariably present in tumor cell lines. Notably, GLI3 was shown as a fragment that was cleaved off from the whole protein during sample preparation. However, its signals represent the true amount of intact GLI3 in the extract. The size of each protein in shown in kDa on the right.

Very peculiar was a varying expression of the ligand sonic Hedgehog among the cell lines, irrespective of the tumor type. This nevertheless does not preclude the efficient functioning of the HH pathway, since, in view of the fact that HH is frequently activated noncanonically at the GLI factors level, the production of the ligand itself (acting by an autocrine or paracrine manner in cell lines) is dispensable. Three cell lines were nontransformed and tested for comparison with tumor cells (HeMN-LP, IMR-90, and WI-38). HeMN-LP (melanocytes) expressed both GLI2 and GLI1, but the two fibroblast cell lines expressed very low GLI2, but retained their GLI1 level. Expression of other components was retained in these normal cell lines. Survivin was present in all tumor cell lines. Our previous results have shown that in IMR90 cells, transfected GLI2 plasmid is capable of evoking the expression of endogenous survivin [9], which underlies the necessity of HH signaling for the survivin expression even in normal cells. BCL-2, another important antiapoptotic protein, was abundantly present in the majority of cell lines, however, in some tumors its expression was completely lacking, independently of the tumor type. Together, the widespread abundance of HH components indirectly support the importance of the HH signaling in tumors and is in accord with the previous results.

2.2. Inhibition of Cell Proliferation by GLI Inhibitor GANT61

We next tested the sensitivity to a GLI inhibitor GANT61 in a panel of 16 tumor cell lines (Figure 2). The tumor types included melanomas, NSCLC and SCLC, osteosarsomas, neuroblastomas, rhabdoid tumors, hepatocellular carcinoma, and pancreatic cancers. Some cells were eradicated completely at the end of the experiment (SK-MEL-3, U-2 OS, MeWo, SK-N-MC, and H196). Another group of cells was only partially sensitive to GANT61 under the experimental conditions (Saos-2, SK-N-SH, G-401, and BxPC-3). The remaining cell lines did not reveal any sensitivity when cultured in GANT61 (A549, Calu-1, A-201, Hep-G2, and the three pancreatic cancer cell lines MIA PaCa-2, PANC-1, and PA-TU-8902). The pancreatic tumors were surprisingly most resistant to GANT61 treatment, although previous reports describe their sensitivity to the blocking of HH signaling [21,60].
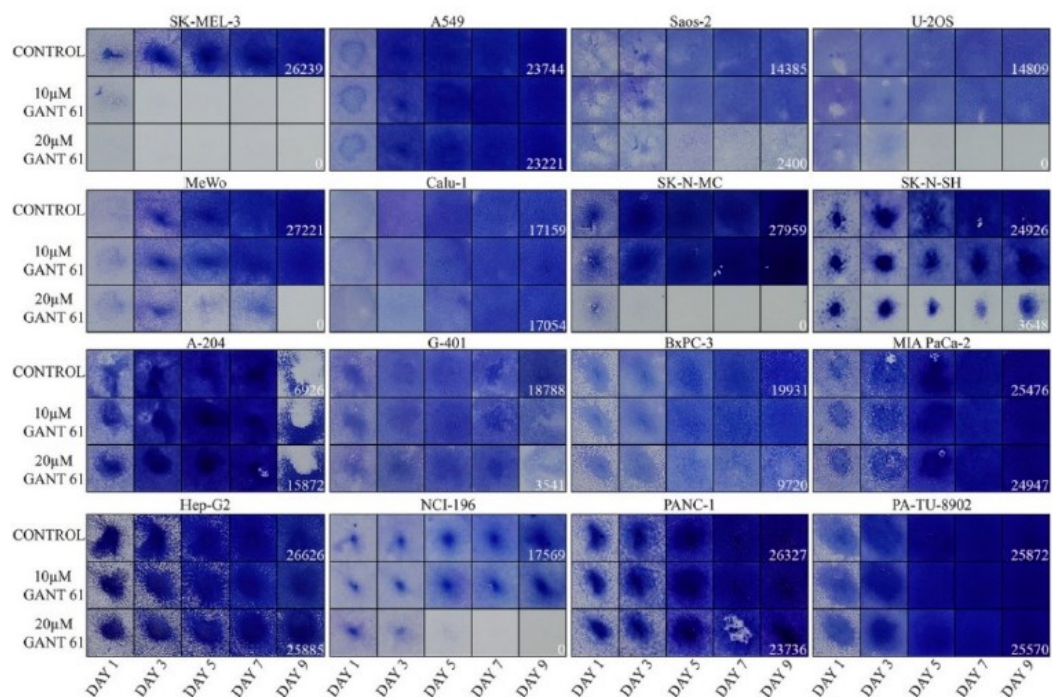

Figure 2. Proliferation assays showing the sensitivity to GANT61. The intensity of staining with crystal violet indicates the relative number of cells. The quantification numbers are given only for day 9 for controls and GANT61 (20 μM) as these fields were the most important outcome of the experiment. Please note that the lower number of A-204 control cells at day 9 is caused by cell detachment. Two experiments with similar results were performed and one is presented. Results are shown as squares cut from the 12-well plate wells.

Expectedly, melanomas were sensitive to GANT61 (Figure 2). We have previously tested melanoma cells and found that GANT61 was variably effective in all tumors. The combination with obatoclax (a BCL-2 family inhibitor) revealed a better effect, showing clear synthetic lethality in six of nine melanoma lines [29] (Figure S1). The most sensitive cell line was SK-MEL-3. Here, less responsive were two osteosarcomas and one SCLC. Also, G-401 was sensitive, but only at day 9. Two neuroblastoma cell lines responded to GANT61 as well. Other cell lines did not reveal any GANT61 sensitivity even after day 9 (A-204, Hep-G2, NSCLC, and pancreatic cell lines from which only BxPC-3 reacted slightly, Figure 2). It is important to note that with the exception of the extremely sensitive SK-MEL-3, all other cells responded only to 20 μM GANT61 and were insensitive to a 10 μM concentration. We can speculate that higher doses of GANT61 or a prolonged time of treatment would have a better effect in eradicating tumor cells. In our assays, longer incubation time was precluded as untreated control cells would overgrow and detach. Our findings suggest that the testing of cancer

cell types might be useful for further consideration of therapy and show that more than half of tested tumors (when we include melanoma cells from Figure S1) were more or less sensitive to 20 μM of GANT61 when observed up to 9 days.

2.3. GANT61 Eradicates Tumor Cells through Apoptosis

To gain insight into the mechanism underlying the eradication of cells in proliferation assays, we carried out the TUNEL assay that detects apoptosis. Many previous papers indicate that GANT61 kills the cells through apoptosis [29,49,57,61]. We have chosen two GANT61-sensitive tumors cell lines, SK-MEL-3 (see Figure 2) and SK-MEL-5 (see Figure S1). Cells were treated with 20 μM GANT61 for 3 days and both detached and attached cells were combined and analyzed using flow cytometry. The extent of apoptosis was analyzed by a TUNEL assay (Figure 3A). The GANT61-treated cells revealed massive apoptosis (reflected by the FITC staining, about 60% of apoptotic cells in SK-MEL-3 and 50% in SK-MEL-5 cells, right peaks, left panels, Figure 3A), while negligible apoptosis was observed in control cells. No cell cycle alteration was seen. We thus presume that no stable blockade of the cell cycle occurred, as the cells stepwise disappeared, although sometimes slowly, which was caused by cell detachment. Since it has been reported that GANT61 may cause autophagy in some cells types [25,62], it can also be possible that in some cell lines, the elimination of cells could be brought about by autophagy. However, it is highly probable that most cells were eradicated by apoptosis as it is a well-known consequence of GANT61 treatment. To corroborate the results in Figure 3A, we left the same cells in a normal medium or medium with 20 μM GANT61 for 3 days, fixed the cells, and mounted in DAPI-containing medium. Apoptotic figures were seen in both cell types, whereas no apoptotic nuclei were present in controls (Figure 3B).
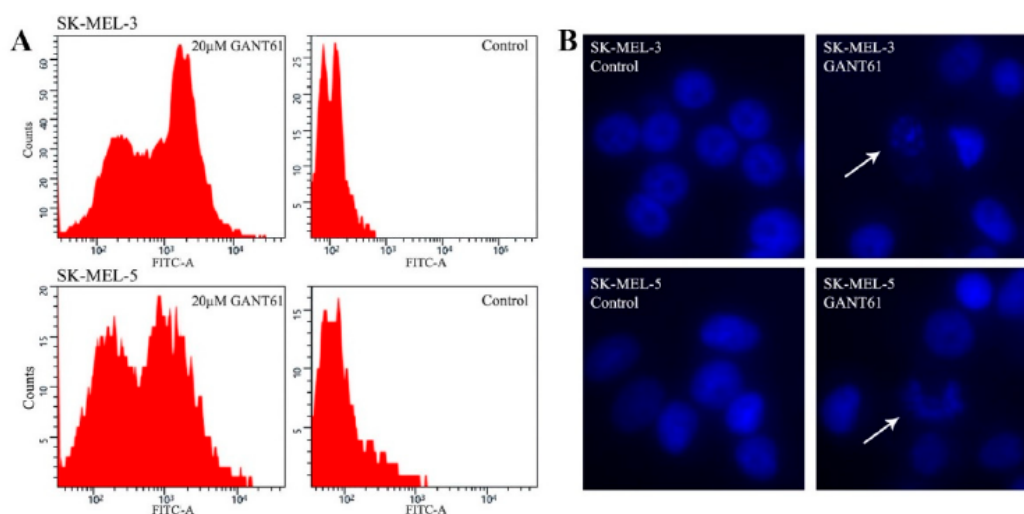

Figure 3. (A) TUNEL assay detecting apoptosis in two cell lines. Cells were seeded on 60-mm dishes, and the next day, 20 μM GANT61 was added. The normal medium was replaced in controls. After three days, the majority of cells treated with GANT61 detached in both SK-MEL-3 and SK-MEL-5 cells. Both detached and remaining attached cells were used for analysis. FITC fluorescence clearly shows massive apoptosis in GANT61-treated cells. The percentage of the apoptotic and nonapoptotic cells were calculated using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The results of cell quantification were as follows. SK-MEL-3 cells treated with GANT61: apoptotic cells 62.62%, nonapoptotic cells 37.38%; SK-MEL-3 controls: apoptotic cells 0.4%, nonapoptotic cells 99.6%. SK-MEL-5 cells treated with GANT61: apoptotic cells 51.97%, nonapoptotic cells 48.03%; SK-MEL-5 controls: apoptotic cells 4.18%, nonapoptotic cells 95.82%. No cell cycle blockade was observed. (B) Fluorescence showing apoptotic nuclei in the same cells as in (A), treated equally with GANT61 or untreated (control cells). Cells were mounted in a medium containing DAPI and documented by fluorescence. Magnification: 200×. Arrows show apoptotic nuclei.

2.4. Activity of the Promoter Containing 12xGLI Consensus Site

To study whether a GLI-responsive promoter-reporter is also affected by HH inhibitors GANT61 and cyclopamine, 12xGLI-luciferase reporter and a reference plasmid were cotransfected in several cell lines that were either variably responsive or nonresponsive to GANT61 in proliferation assays. As shown in Figure 4, the sensitive SK-MEL-3 cells were inhibited by cylopamine and GANT61 extensively. To a lesser extent, reporter activity in G-401, A-204, and U-2 OS was also inhibited. Of these cells, U-2 OS were eradicated from day 5 onwards in the proliferation assay, G-401 were diminished only on day 9, and A-204 were resistant in the proliferation assay (Figure 2). The inhibition of the reporter by GANT61 or cyclopamine was insignificant in other cell lines (PANC-1, PA-TU-8902, MIA-PaCa-2, A-549, and Hep-G2). These cells were also completely resistant in the proliferation assay (Figure 2). The reporter activity thus approximately mimicked the sensitivity of cells to GANT61 (A-204 cells were only negligibly, though significantly, inhibited by cyclopamine, due to very low +SD, and were resistant to GANT61 in proliferation assay). Together, the results indicate a correlation between the sensitivity to inhibitors in the reporter assay and the sensitivity to GANT61 in longer proliferation analysis.
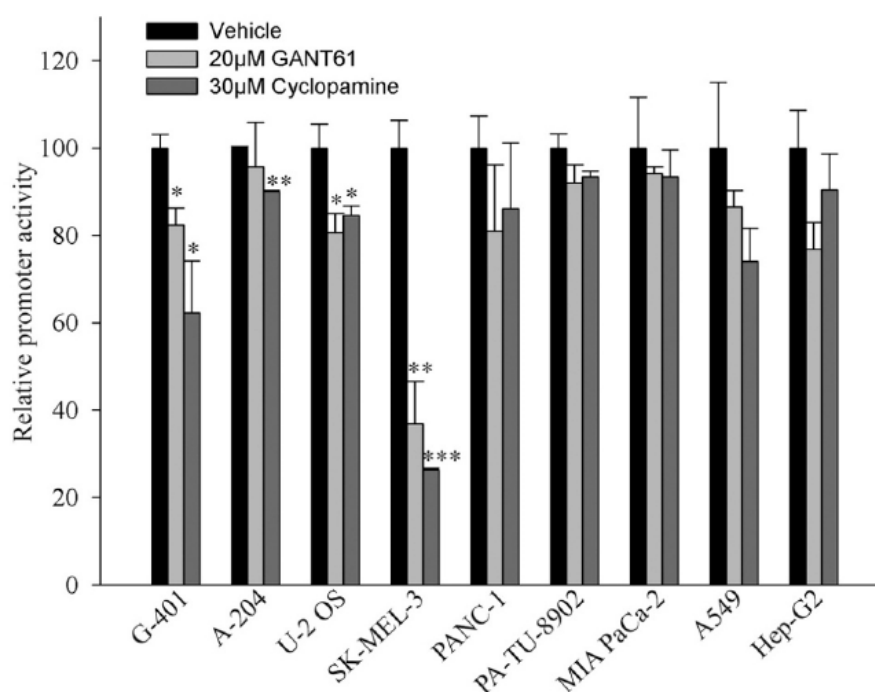
Figure 4. GANT61 and cyclopamine slightly reduced the 12xGLI reporter activity. Cells were seeded in 12-well plates and transfected the next day with the 12xGLI-luciferase plasmid together with a *Renilla luciferase* plasmid for the correction of transfection efficiency. The next day, inhibitors were added to the indicated concentration and cells were harvested 20 h later. No cell deterioration was observed after this period, even in sensitive SK-MEL-3 cells. The experiment was performed twice in triplicates with similar results and one experiment is presented. Data are presented as mean + SD. No mark means insignificant, statistical significance is: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Discussion

The HH signaling pathway, acting through transcription factors GLI1, GLI2, and GLI3, has been identified as critical for the initiation and progression of a number of cancers. Originally, it was believed to be important for only basal cell carcinoma (BCC) and medulloblastoma. Gradually, the pathway becomes a crucial signaling pathway for all frequent cancer types with the GLI family transcription factors being essential in tumor initiation, progression, EMT, CSC, and metastasis, dependent on the

tumor cell context. HH signaling is a network rather than as a simple linear pathway because of its cooperation with many other cell signaling pathways and its frequent noncanonical activation. GLI factors have several oncogenic targets [63]. Recently, using a large tumor panel, we identified survivin as another important GLI2 target in more than half of tumor cell types [9], suggesting a synergy in HH and survivin in forming tumors stemness and maintaining CSC. This implies more effective therapy by combining HH and survivin inhibitors.

Here, we have first analyzed the expression of HH cascade components across a panel of 56 tumor types using Western blot analysis. It was found that they are generally expressed (only exceptionally showing lower expression level). Importantly, either GLI1 or GLI2 were always present in all samples. In three normal control cell lines, the HH proteins were also present. HH signaling is emerging to be essential for the progression of nearly all tumors [12,13]. The presence of its components is therefore required for the proper progression of the pathway. In proliferation assays, GANT61 was active in melanoma cells (Figure 2 and Figure S1) and also in several other tumor cell lines. The most resistant seemed to be NSCLC and pancreatic cancer cells. This was rather surprising as many reports describe the blockage of the HH pathway in the treatment of pancreatic cancer in preclinical and clinical settings. In tumors, the dense impenetrable stroma is mixed with the pancreatic cancer cells in vivo, due to which, drugs cannot invade across this physical barrier, and that may cause a drug resistance [22,64–66]. Since in cell lines the stroma is missing, the drugs should have better access to tumor cells and the druggability might be more feasible. As GANT61 appeared to be nonfunctional in eradicating pancreatic tumor cells, the HH pathway possibly needs, e.g., a second agent to achieve cell killing. A possible explanation could also be that the cell lines used here have not been sensitive to GANT61, while other cell lines (not tested) might have been responsive. In pancreatic tumors, the situation might be even more complicated, e.g., because stromal cells themselves produce Hedgehog and HGF that support the tumor growth [67]. It requires further clarification why in pancreatic cancer the HH pathway sensitivity to drugs in vivo has specific requirements in which tumor stroma is determining, causing the known resilience and drug resistance of these tumors.

Our results suggest which type of cancer is resistant or sensitive to GANT61 when it is applied directly on cells in culture (Figure 2). Malignant melanomas are sensitive, when taken into account also our previous results (Figure S1). Thus, GLI factors are important to contribute to keeping their antiapoptotic status. It is believed that MITF (microphthalmia-associated trancription factor), a key factor in melanoma transcription circuitry, maintains antiapoptosis in melanomas [68]. It has been nevertheless demonstrated that low-MITF melanoma cell lines can also proliferate very fast, implicating sufficient antiapoptotic protection [29,69]. HH-GLI signaling has been recognized to keep melanoma stemness and maintain the presence of CSC [70]. Furthermore, the two neuroblastoma cell lines and one SCLC cell line were also relatively sensitive to GANT61, whereas two NSCLC were resistant. In GANT61-resistant cells, antiapoptotic signals ensuring tumor progression can maintain apoptosis by other pathways. Reporter assays measuring the sensitivity of the 12xGLI consensus promoter to GANT61 and cyclopamine roughly correlated with cell proliferation. Our results suggest that HH signaling participates in preventing cell death perhaps in more than half of all tumors cell lines. We speculate that the situation might be similar in other tumor cell lines as well. Taken together, HH signaling plays an important role in preventing tumors cell apoptosis in some cancer cell types.

4. Materials and Methods

4.1. Cell Cultivation

Cells were maintained in appropriate media (DMEM or RPMI1640) supplemented with 10% fetal calf serum (Gibco, Waltham, MA, USA), L-glutamine, streptomycin, and penicillin (Sigma, St. Louis, MO, USA). Some cells were cultured in EMEM medium supplemented also with essential amino acids and pyruvate (Sigma). Fresh media were replaced every third day. HH inhibitors GANT61 or cyclopamine were present in media as indicated in Figures and Figure legends. All melanoma

cell lines were maintained in RPMI1640 medium with the exception of lines WM35 and WM1552C that were kept in DMEM. NSCLC and SCLC cell lines cells were grown in RPMI1640 medium with the exception of Calu-1 (DMEM). SK-N-SH, SH-N-MC, HT-1080, and T98G cells were maintained in EMEM. The remaining cell lines were grown in DMEM medium.

4.2. Cell Lines

All cell lines were of human origin. Melanoma cell lines DOR, Beu, and Hbl were previously described [29]. Other melanoma cell lines (MeWo, SK-MEL-2, SK-MEL-28, SK-MEL-5, SK-MEL-3, Malme 3M, HT144, WM35, WM1552C, and RPMI-7931) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Normal human melanocytes HeMN-LP were from Cascade Biologics (Portland, OR, USA). NSCLC lung cancer cell lines A549, HT1299, A-427, Calu-1, H-460, H-520, H596, H-661, H-2170, and SK-MES-1, and SCLC cell lines H-446, H-69, H-209, H-82, H-345, H-146, H-378, H-196 were purchased from ATCC. 293FT cells were from Invitrogen (Carlsbad, CA, USA). Colorectal cell lines LoVo, SW480, HCT116 were from ATCC. All other cell lines were purchased also from ATCC: G-401 and A-204 (rhabdoid tumors), U-2 OS and Saos-2 (osteosarcomas), HeLa S3 and C33A (cervical carcinomas), 293 (renal carcinoma), HT-1080 (connective tissue fibrosarcoma), SW-13 (adrenal gland carcinoma), T98G (glioblastoma), IMR90 and WI-38 (normal human fibroblasts), Jurkat (T-cell leukemia), Hep-G2 (hepatocellular carcinoma), SK-N-SH and SH-N-MC (neuroblastomas), PANC-1, PA-TU-8902, MIA PaCa-2, and BxPC-3 (pancreatic carcinomas).

4.3. Western Blots

Commercially available primary antibodies used were as follows: Sonic Hedgehog, Cell Signaling Technology #2207 (Danvers, MA, USA); Patched, Biorbyt #157169 (San Francisco, CA, USA); SMO, #ab72130 (Abcam, Cambridge, UK); SuFu, Cell Signaling #2520; Gli, Abcam #ab134906; Gli2, #sc-271786 (Santa Cruz Biotechnology, Dallas, TX, USA); Gli3, Biorbyt #157158; Survivin, Santa Cruz #sc-17779; BCL-2, BD Pharmingen #556354 (San Jose, CA, USA); β-actin, Sigma #A5316. HRP-labelled second antibodies were from Cell Signaling.

For Western blot analysis, cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS), supplemented with aprotinin, leupeptin, pepstatin (Sigma), COMPLETE, and PhoStop (Roche, IN, USA). Total lysates containing 30 μg of protein were separated on SDS-PAGE gels and subsequently transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). Membranes were then subjected to probing with antibodies. Western blot signals were detected by using SuperSignal West Pico Chemiluminescent substrate (Fisher Scientific, Waltham, MA, USA) and exposed on films.

4.4. Proliferation Assays

To perform proliferation assays, colony outgrowth assays were carried out. Cells were trypsinized and seeded in about 40–50% confluency on 12-well plates (day 0). The next day (day 1), cell lines were treated with 10 μM GANT61 or 20 μM GANT61 (SelleckChem, München, Germany), for a maximum of 9 days. The medium was refreshed every third day. The plates were then fixed in 3% paraformaldehyde solution in 1× PBS and stained with 1% crystal violet and photodocumented. Two most important fields (day 9, control and 20 μM GANT61) were quantitated using ImageJ software. Two experiments were performed in duplicate. Results of both experiments were similar.

4.5. Detection of Apoptosis

A TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling) assay was performed according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). Results of FITC staining were analyzed on a flow cytometer BriCyte EA (Mindray, Shenzhen, China). A total of 50,000 cells were analyzed in each sample. The number of apoptotic cells was determined using ImageJ software.

4.6. Microscopic Detection of Apoptotic Nuclei

Immunofluorescence assays were performed as described previously [71]. Briefly, cells were seeded in NUNC (Roskilde, Denmark) chambers, 20 μM GANT61 added next day and treated (or untreated, controls) for three days, and mounted in a DAPI-containing medium. Images of nuclear apoptotis figures and controls were there taken using a fluorescent microscope.

4.7. Reporter Assays

Luciferase Reporter Gene Assay: Luciferase reporter plasmid with luciferase gene under the transcriptional control of 12xGLI full consensus was obtained from Prof. R. Toftgard (Karolinska Institutet, Stockholm, Sweden). After transfection of the plasmid (1 μg), together with the *Renilla luciferase* reporter plasmid (as a reference for transfection efficiency) on the 12-well plates in triplicates, the inhibitors GANT61 and cyclopamine were added at concentrations indicated in Figure 4 for 20 h. Cells were then harvested and the reporter activity was measured using a dual luciferase kit (Promega, Madison, WI, USA) according to the instructions of the manufacturer. Statistical significance is shown in the Figure 4. Two experiments were performed and one is presented. Results of both experiments were similar.

4.8. Statistical Analysis

To calculate the statistical significance of the reporter assays, a two-tailed Student test was used. The *p* values are listed in the corresponding figure legend. In all figures the error bars represent mean + SE. Proliferation assays and TUNEL assay were quantified by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Abbreviations

CSC	cancer stem cells
HRP	horseradish peroxidase
DAPI	40,6-Diamidino-2-Phenylindole, Dihydrochloride
SMARCB1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1
EMT	epithelial-to-mesenchymal transition
TUNEL	terminal deoxynucleotidyl transferase-mediated d-UTP Nick End Labeling
AR	androgen receptor
BCC	basal cell carcinoma
GLI	glioma family zinc finger protein
MITF	microphthalmia-associated trancription factor
HGF	hepatocyte growth factor
PTCH	patched
SMO	smoothened, frizzled class receptor
NSCLC	Non-small cell lung cancer
SCLC	Small cell lung cancer
SWI/SNF	SWItch/Sucrose Non-Fermentable
mTOR	mechanistic target of rapamycin
FITC	fluorescein isothiocyanate

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4.4 PUBLICATION IV

Inducibly decreased MITF levels do not affect proliferation and phenotype switching but reduce differentiation of melanoma cells

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Abstract

Melanoma arises from neural crest-derived melanocytes which reside mostly in the skin in an adult organism. Epithelial–mesenchymal transition (EMT) is a tumorigenic programme through which cells acquire mesenchymal, more pro-oncogenic phenotype. The reversible phenotype switching is an event still not completely understood in melanoma. The EMT features and increased invasiveness are associated with lower levels of the pivotal lineage identity maintaining and melanoma-specific transcription factor MITF (microphthalmia-associated transcription factor), whereas increased proliferation is linked to higher MITF levels. However, the precise role of MITF in phenotype switching is still loosely characterized. To exclude the changes occurring upstream of MITF during MITF regulation *in vivo*, we employed a model whereby MITF expression was inducibly regulated by shRNA in melanoma cell lines. We found that the decrease in MITF caused only moderate attenuation of proliferation of the whole cell line population. Proliferation was decreased in five of 15 isolated clones, in three of them profoundly. Reduction in MITF levels alone did not generally produce EMT-like characteristics. The stem cell marker levels also did not change appreciably, only a sharp increase in SOX2 accompanied MITF down-regulation. Oppositely, the downstream differentiation markers and the MITF transcriptional targets melastatin and tyrosinase were profoundly decreased, as well as the downstream target livin. Surprisingly, after the MITF decline, invasiveness was not appreciably affected, independently of proliferation. The results suggest that low levels of MITF may still maintain relatively high proliferation and might reflect, rather than cause, the EMT-like changes occurring in melanoma.

Keywords: melanoma ● MITF ● phenotype switching ● proliferation ● invasiveness ● differentiation

Introduction

Malignant melanoma is an aggressive tumour of neuroectodermal origin that has a dismal prognosis if it is not excised at an early stage. More than 50% of melanoma cases harbour the BRAF(V600E) mutation [1, 2]. However, singular targeted inhibition of BRAF leads invariably to acquired resistance (which can be also inherent) that can result in worsening of the patient's prognosis also through inducing the therapy-induced pro-oncogenic secretome [3]. Melanoma cells are very early phenotypically diversified and undergo phenotype switching resembling the EMT, through which they acquire considerable micro-heterogeneity resulting in plasticity, capability of invasion and migration. These properties lead to metastasis and poor prognosis [4–7]. EMT is mostly a reversible process through which undergoes epithelial tumours to gain the mesenchymal phenotype and more oncogenic characteristics but occurs also in non-epithelial cancers [8, 9].

Melanocyte-specific isoform of MITF (microphthalmia-associated transcription factor) is a pivotal protein determining the melanocyte lineage identity and conferring a strong antiapoptotic activity to melanoma cells [10]. This is accomplished through the direct activation of expression of several antiapoptosis factors such as BCL2 [11], livin [12], BPTF [13] and others.

Two phenotypically distinct populations of melanoma cells were described related to MITF levels: High-MITF population is associated with differentiation and proliferation, whereas low-MITF cells, although they proliferate slowly, are endowed with the invasive and EMT-like characteristics [14], and they express pro-oncogenic genes such as Brn2 [15–19], GLI2 [20], JARID1B [21], Axl [22] and others. On the other hand, it has been found that a large proportion (over 25%) of melanoma cells derived directly from patients are capable of forming tumours in highly immunocompromised NOD/SCID

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interleukin-2 receptor gamma chain null (Il2rg(−/−), NSG) mice [23]. Also, the phenotypic heterogeneity in melanoma is extremely reversible and not hierarchically organized [24]. These findings substantially challenge the concept of a small population of rare cancer initiating cells with stem cell (SC) properties [21, 25, 26] which are recruited from the invasive cells and have a high self-renewal potential and propensity to form metastasis.

When studying the phenotypic changes in melanoma, it is crucial to discern the effects of MITF alone from the effects of expression changes in many MITF transcriptional regulators and cofactors that operate upstream of MITF. They undoubtedly influence not only MITF but also many other targets involved in the phenotype outcome *in vivo*. Events caused purely by MITF down-regulation can be achieved through manipulating MITF levels alone, an approach that is not feasible to perform *in vivo*. It is thus highly desirable to understand precisely the mechanisms which MITF plays in modulating tumour cell invasiveness, plasticity, migration, proliferation and metastasis *in vitro* and *in vivo*.

We used here the doxycycline (DOX)-based inducible lentiviral system to stepwise decrease MITF level in six melanoma cell lines. In this setting, the expression of upstream genes regulating MITF expression remained intact, simplifying the interpretation of phenotype changes and evaluation of the effect of exclusive down-regulation of MITF. We found no profound changes in proliferation of whole cell populations, EMT gene expression pattern and invasiveness. In contrast, the expression of the downstream differentiation markers melastatin and tyrosinase and the antiapoptotic MITF target livin diminished after DOX-dependent reduction in MITF protein level. Based on these experiments with cell lines, we suggest slightly modified model concerning the role of MITF in proliferation and invasiveness of melanoma cells. The data further suggest that more complex events may occur during the phenotype switching in melanoma that might be a more non-uniform process than previously anticipated and may be a cause (rather than a result of) of the low-MITF levels in the invasive subpopulations.

Materials and methods

Cell culture

Melanoma cell lines SK-MEL-3, SK-MEL-5, SK-MEL-28, Malme 3M and MeWo were purchased from ATCC and were grown using EMEM complete medium with non-essential amino acids and pyruvate, or RPMI1640 medium (for Malme 3M). 501mel cells were generously provided by Dr. R. Halaban (Yale University) and maintained in RPMI1640 medium. All media were supplemented with 10% FCS and antibiotics. All cell lines harbour mutated BRAF(V600E), with the exception of MeWo cells which are BRAFwt; 293FT cells were purchased from Invitrogen (Carlsbad, CA, USA) and cultivated in DMEM with 10% FCS.

Proliferation assays

Colony outgrowth assay

After culturing the cells 6 days in appropriate concentration of DOX (Invitrogen), cells were seeded at low density in 12-well plates and grown for 9 days. The medium with or without (as a control) DOX was changed every other day. Cells were then fixed, stained with crystal violet and quantified.

Growth curves

This experiment reflects the cell growth after previous long-term cultivation in DOX. Cells were first maintained for 5 weeks in appropriate DOX concentration, then plated in 24-well plates at low density and fixed on days 0, 3, 6 and 9. Medium was changed every other day. Cells were fixed, stained with crystal violet, destained and quantitated on a spectrophotometer. Growth curves were constructed using the triplicate data. The levels of MITF in DOX remained decreased all the time as assessed by Western blot. Curves are shown with a standard error for each point.

Western blot analysis and immunofluorescence

Cells were lysed in a complete RIPA buffer (1% NP-40, 150 mM NaCl, 5 mM EDTA, 0.5% sodium deoxycholate, 50 mM Tris-HCl pH 7.5, 0.1% SDS) with added protease and phosphatase inhibitors aprotinin, leupeptin, pepstatin, phenylmethylsulphonylfluoride and PhosStop (Roche, Indianapolis, IN, USA). After the electrophoresis on 10–12% SDS–polyacrylamide gels, the proteins were transferred onto PVDF membrane (Millipore, Billerica, MA, USA). Blots were incubated with primary and horseradish peroxidase-conjugated secondary antibodies and detected by chemiluminescent determination. The following commercially available antibodies were used for Western blots: antibody against MITF (cat. no. MS-772; Neomarkers, Fremont, CA, USA), BCL2 (556 354; Becton Dickinson, San Diego, CA, USA), livin (sc-30161; Santa Cruz Biotechnology, Dallas, TX, USA), Axl (sc-166269; Santa Cruz), β-catenin (8480; Cell Signaling, Danvers, MA, USA), SRC (2109; Cell Signaling), β-actin (A5316; Sigma-Aldrich, St Louis, MO, USA), E-cadherin (3195; Cell Signaling), N-cadherin (13116; Cell Signaling), SLUG (9585; Cell Signaling), SNAIL Santa Cruz, sc-28199), vimentin (5741; Cell Signaling), ZEB1 (3396; Cell Signaling), ZEB2 (sc-271984; Santa Cruz), p27 (Santa Cruz, sc-528), KLF4 (LS-C415468; LSBiotechnologies, Seattle, WA, USA), ALDH1A1 (LS-B10149; LSBiotechnologies), Brn2 (sc-393324; Santa Cruz), SOX2 (5024; Cell Signaling), OCT4 (sc-514295; Santa Cruz). For immunofluorescence, cells were fixed in 3% paraformaldehyde the next day after seeding, permeabilized and stained with anti-MITF antibody followed by a FITC-labelled second antibody. Cell chambers were then mounted in the mounting medium with DAPI.

Lentivirus production and infection of target cells

ShRNA-coding hairpin sequence against MITF [27] was cloned in DOX-inducible (Tet-On) Tet-pLKO-puro plasmid [28] (Addgene plasmid no. 21915). This shRNA sequence has been previously verified and down-regulates MITF level best among other tested sequences. Lentiviruses were packaged in 293FT cells as described earlier [29]. Plasmid with scrambled shRNA sequence was used as a control. Six melanoma cell lines (above) were infected with the fresh virus overnight in the presence of 6 μg/ml Polybrene (Sigma-Aldrich) and then briefly (4–5 days) selected in puromycin (Sigma-Aldrich) and maintained in low puromycin (0.25 μg/ml) media.

Invasivity and wound-healing assay

For these assays, cells were grown for 6 days in medium without DOX and with 1 μg/ml DOX (or in 0.5 μg/ml DOX for wound-healing assay). Estimation of cell invasiveness has been made using the collagen invasivity kit (Millipore). For the wound scratch migration assays, cells were prepared in duplicates on 12-well plates. Next day, cells were near-confluent and wounded using 1-ml sterile pipette tip and photodocumented for control time zero, washed repeatedly and starved for 24 hrs in medium containing 0.5% FCS. Next day, cultivation medium containing 15% FCS was added (still keeping the cells with or without DOX), and invasion of cells in the same areas as at the time zero was photodocumented after next 24 and 48 hrs.

Viability

Cell viability was estimated on cells in duplicates. Cells bearing the inducible shRNA against MITF or control cells were treated for 6 days with the indicated concentrations of DOX, replated onto 12-well plates, and viability was determined next day by the MTT viability kit (Sigma-Aldrich) according to the manufacturer's instructions.

Real-time PCR

Estimation of melastatin mRNA levels was performed with primers and a labelled probe as described in the original procedure [12]. Primers for estimation of tyrosinase were as follows: forward, 5′- CCAGAAGCTGA CAGGAGATG; reverse, 5′- AGGCATTGTGCATGCTGCTT; probe, 5′- FAM-ACGGCGTAATCCTGGAAACCATGACA-TAMRA. After total RNA was isolated using TRIzol (Life Technologies, Carlsbad, CA, USA), 2 μg of RNA was reverse-transcribed using transcriptor reverse transcriptase (Roche), and cDNAs for melastatin and tyrosinase were quantitated using Taqman system QuantiTect Probe PCR Kit (Qiagen, Hilden, Germany). Data were acquired on a ViiA7 system (Life Technologies). Each experiment has been performed twice with similar results. Data are presented after the compensation to β-actin mRNA levels as a control gene.

Statistics

Each experiment was performed at least two times with consistent results. Data in graphs are presented as means and their standard errors. Statistical significance was determined using the Student's t-test. P value <0.05 or <0.01 was considered statistically significant as indicated. For quantification of proliferation assays, the ImageJ software (National Institutes of Health, Bethesda, MD, USA) was employed, and one of two experiments is presented.

Results

Reduced MITF levels do not cause halt of proliferation or cell cycle arrest

We generated lentivirus encoding shRNA-MITF enabling the regulatable decrease in MITF levels in cell lines. This approach enables the elimination of MITF upstream events that occur *in vivo*, which downregulate MITF but can have many other activities which are MITF-independent. Thus, in our system, only MITF-regulated genes are participating in the resulting phenotype (schematic Fig. 1A).

Cells infected with the Tet-pLKO-puro-based produced virus were selected in puromycin and constituted DOX-responsive cell lines with gradually decreased MITF levels after increasing DOX doses. Six cell lines with high or average MITF levels were chosen to better follow the stepwise MITF depletion. We used 1 μg/ml of DOX as the highest concentration because higher DOX began to cause a non-specific toxic effect to the cells. MITF levels decreased gradually with increasing DOX doses in all cell lines tested (Fig. 1B). No change in MITF level was seen in control virus-infected cells, as exemplified in three cell lines (Fig. 1C). Although MITF protein was substantially decreased (Fig. 1B), high exposures revealed still appreciable levels even in 1 μg/ml of DOX (Fig. 1F). This is in contrast with our previous results where we were able to ablate MITF completely (targeting the same sequence) with transfected non-inducible pSUPER-puro-shMITF plasmid and puromycin selection [27] in 501mel cells (Fig. S1), which was followed by cell cycle arrest and subsequent apoptosis. The difference in results is apparently due to the different silencing system and a very effective block of MITF expression when shRNA was cloned in pSUPER plasmid and transfected. To substantiate the knockdown, we verified the decreased MITF levels and assessed the results by immunofluorescence. MITF staining was decreased in all DOX-treated cells but not in controls (Fig. S2).

Surprisingly, even the highest decrease in MITF had relatively little effect on cell proliferation in this study, as assessed by colony formation assay (Fig. 1D). Evidently, a smaller decrease in proliferation was seen in most cell lines at high DOX, but a very slight retardation of growth was visible also in some controls (Fig. 1E). Collectively, reduction in MITF levels had no dramatic effect on proliferation rate in melanoma cell lines, probably partly because the degree of knockdown left some MITF level which was sufficient for proliferation. Consistent with this, little or no changes in the cell cycle profiles were observed in cells without DOX or containing 1 μg/ml of DOX (Fig. S3). The data thus show that even small

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Fig. 1 Gradually decreased MITF protein in the inducible system causes minimal changes in proliferation. (**A**) A scheme of experimental setting with a view of groups of MITF-inducible genes. (**B**) Infection of six melanoma cell lines with a lentivirus carrying the shRNA sequence directed to MITF, followed by a brief puromycin selection. Incubation of cells in increasing concentrations of DOX leads to a stepwise disappearance of the MITF Western blot signal. The Western blot was performed 6 days after incubation without or with DOX. (**C**) No MITF level changes were seen in control virus-infected cultures. (**D**) Proliferation rates are determined in increasing DOX concentrations. Cells were maintained in DOX for 6 days, and then, the experiment was carried out and quantitated by ImageJ. Two experiments with consistent results were performed and one is presented. (**E**) Similar to **D**, control virus-infected cultures grew in all DOX concentrations. The setting of the experiment was the same as in **D**. (**E**) Longer exposure of the same Western blots as shown in **B**. Only two highest DOX concentrations are shown. In all cell lines, some residual MITF remains even in the highest DOX concentration.
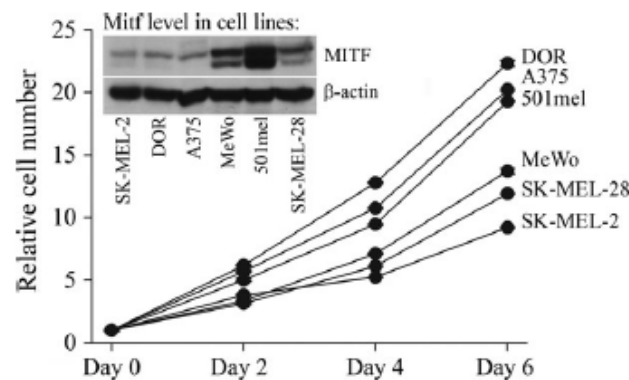
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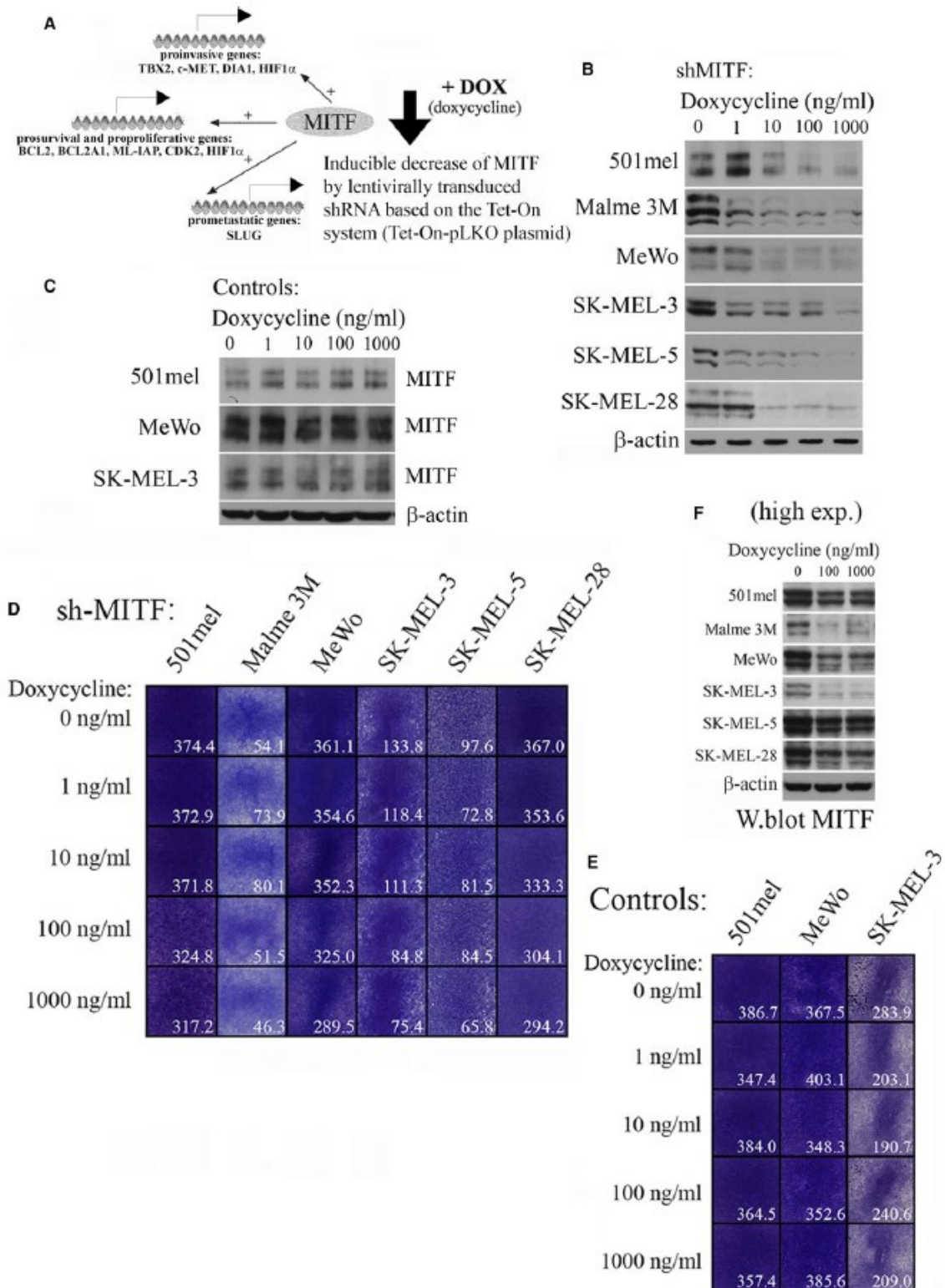
amounts of MITF are capable of maintaining proliferation of melanoma cells.

To test the growth of established melanoma lines, we chose three low-MITF and three high-MITF cell lines and determined their proliferation rate. There was no relationship between the MITF level and proliferation (Fig. 2). The growth rate data of low-MITF cells (SK-MEL-2, Dor and A375) were completely mixed with the data of high-MITF cell lines (MeWo, 501mel, SK-MEL-28), indicating that even low level of MITF can sustain high proliferation rate in some melanomas, apparently dependent on the cellular context.

Inducible reduction in MITF protein generally does not induce the phenotype switching towards EMT changes or expression of SC markers

Although MITF levels are not critical for proliferation either in an artificial inducible system or in native cell lines (above), the presence of MITF is essential to prevent apoptosis in melanoma cells [10, 27, 30]. Furthermore, low-MITF populations of cells are believed to proliferate slowly but to be highly invasive, while high-MITF cells are proliferating rapidly and are not invasive. This 'rheostat model' has been proposed first in 501mel cells [14]. As invasive cells undergo EMT-like changes, we studied whether the inducible MITF decrease *per se* could induce EMT hallmarks. The EMT-like changes in melanoma are characterized by the increased expression of markers such as SNAIL, ZEB1, N-cadherin, vimentin and decreased E-cadherin [31, 32]. We first determined expression levels of proteins previously reported to be important for melanoma progression (Fig. 3A). We found no change in SRC and β-actin as controls. Also β-catenin did not display any changes. BCL2, a MITF target, did not decrease as well (only slightly in SK-MEL-28). Axl level increased in MeWo but remained unchanged in Malme 3M upon
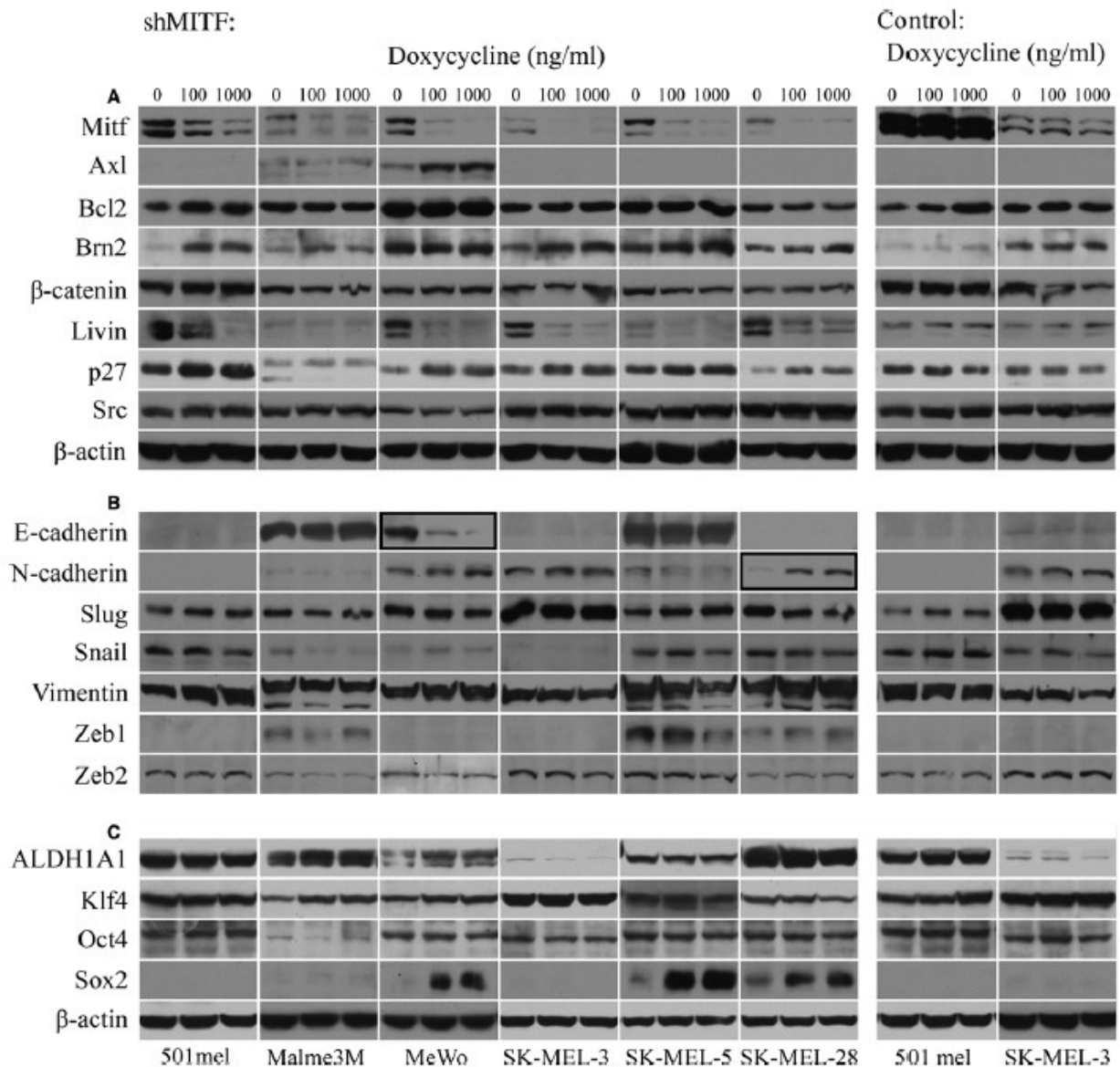
DOX addition and was not present in other cell lines. On the other hand, livin perfectly mimicked the down-regulation of MITF (Fig. 3A) as it is a known MITF downstream target. P27 protein was found increased after increasing DOX levels in three cell lines, very slightly increased in two lines and remained unchanged in one line (Fig. 3A). This cdk inhibitor has been originally described to be the cause of inhibition of proliferation in pro-invasive subpopulations [14, 33]. Brn2 protein appeared increased in MITF-lowered samples in four cell lines, most prominently in 501mel cells (consistent with the original model [14]), while it remained unchanged in two cell lines (Fig. 3A).

As a next step, we have estimated markers which should undergo changes during the EMT-like process after the MITF decrease. We analysed protein levels of vimentin, E-cadherin, N-cadherin, SLUG, SNAIL, ZEB1 and ZEB2. ZEB2 and SLUG are mostly considered to be pro-proliferative and pro-differentiative markers, not involved in the EMT process in melanoma) and revealed a pattern showing only minimal changes (Fig. 3B). The only two characteristic pictures typically reflecting EMT were the decrease in E-cadherin in MeWo cells and increase in N-cadherin in SK-MEL-28 cells. Vimentin was uniformly expressed with increase in 501mel and SK-MEL-28 cells and a low decrease in SK-MEL-3 cells. Further, many EMT-related proteins were absent from cells at all DOX concentrations (*e.g.* E-cadherin and ZEB1 were absent in three different cell lines). In aggregate, lowering of MITF levels alone generally does not lead to EMT-like phenotype patterns on Western blots in six melanoma cell lines.

We examined also the pattern of cancer SC markers after MITF down-regulation. We found no change in the level of proteins ALDH1A1, KLF4 and OCT4 (Nanog was negative in all cell lines, not shown), whereas a profound increase in SOX2 was observed at both DOX concentration in SK-MEL-3, SK-MEL-5 and SK-MEL-28 cell lines. Other three lines did not express SOX2 (Fig. 3C). Thus, for a high expression of SC marker SOX2, which is critical for forming the tumour-initiating cells in melanoma [34], low-MITF level is required. This finding is consistent with the accepted model that the melanoma SC is recruited from the invasive low-MITF populations.

The growth rate of the whole cell population remains unchanged in low-MITF long-term cultures

As the proliferation assays after several days in DOX did not show any substantial growth diminution, we reasoned that longer cultivation of cells in DOX could be required to achieve the effect of more prominent growth deceleration. The cell lines were cultured for 5 weeks with or without DOX, and the proliferation curves were determined during next 9 days. The same experiment was also performed with control virus-infected cells to exclude the possible non-specific effect of DOX at the highest concentration. No substantial changes were observed when proliferation of pooled cultures cultivated in media −DOX and +DOX was estimated (Fig. S4A); 501mel

Fig. 2 The proliferation rates of native three low-MITF and three high-MITF melanoma cell lines are completely intermingled. Cells were seeded at lower density in triplicates and were fixed every other day. There were only minimal changes among triplicates, as demonstrated by extremely small S.E. values. Insert: Western blot stained with the anti-MITF antibody shows the MITF protein levels in cell lines analysed and equal loading (β-actin).

Fig. 3 Pattern of gene expression after down-regulating MITF by two highest DOX concentrations. (**A**) Western blot of proteins not directly connected with the EMT-like process. Livin is a MITF target and mirrors the decrease appearing in MITF samples. Axl is negatively correlated with MITF only in MeWo (the only BRAFwt cell line), where it is most prominently expressed. Actin control shows equal loading. (**B**) The proteins which are often associated with EMT. Two triplets of typical EMT changes (E-cadherin in MeWo and N-cadherin in SK-MEL-28) are framed. Loading and sample's integrity are demonstrated by expression by SLUG and vimentin expression. (**C**) Stem cell markers expression. Two control virus-infected cell lines are also shown (right). Some proteins (*e.g.* Axl or SOX2) were expressed only in some cell lines. All cells were maintained in DOX for 6 days before performing the Western blots.

cells +DOX ceased to grow at the end of the experiment, probably because their proliferation is highly dependent on MITF [27]. Proliferation of some cell lines was slower even from the day 3 onwards, but this phenomenon was seen also in controls (MeWo and MeWo control, SK-MEL-3 and SK-MEL-5 control). Control Western blots

confirmed lower MITF in DOX-containing cultures after the long-term cultivation (Fig. S4B). Together, the maintenance of melanoma cells in up to 1000 ng/ml DOX did not have any great deleterious effect on the rate of long-term proliferation in pools of infected cell lines.
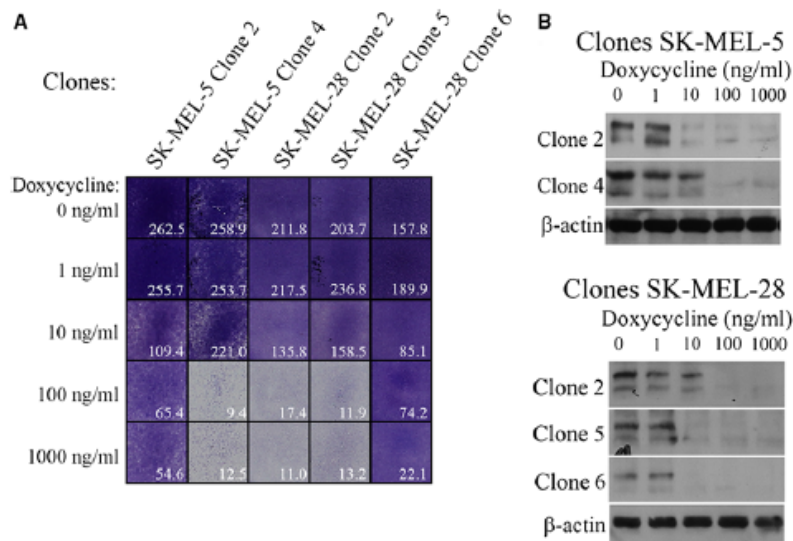
Fig. 4 Cell proliferation of five isolated and expanded clones. (**A**) Of 15 isolated clones from cell lines SK-MEL-5 or SK-MEL-28, maintained in DOX for 4–5 weeks required for expansion, only five clones (shown) revealed prominent decrease in growth in colony outgrowth assay. Two identical experiments gave similar results, and one experiment is depicted. Other 10 clones resembled minimal proliferation changes comparable to Figure 1D (not shown). Proliferation pictures were obtained during 9-day incubation in appropriate DOX concentration. After the removal of DOX, the slowdown clones recovered to near-to-normal proliferation rate. (**B**) Confirmation of stepwise MITF protein diminution on Western blots after DOX treatment in the five clones used in **A**, performed at the beginning of the proliferation experiment.

In long-term cultures, the minority of individual clones with reduced MITF reveals slow proliferation

Given the proliferation of the whole cell population was only slightly affected by MITF decrease, we investigated whether the growth of cultures raised from the individual cell clones could be retarded in DOX. To this end, we isolated and expanded 15 randomly chosen individual clones from SK-MEL-28 or SK-MEL-5 cell lines and maintained them in 0, 100 or 1000 ng/ml DOX concentrations for 5 weeks. The proliferation was determined thereafter by the colony outgrowth assay. We found five (of 15) clones that were substantially retarded in proliferation in these low-MITF cultures. The three most retarded clones were two SK-MEL-28 clones and one SK-MEL-5 clone (Fig. 4A). These three expanded clones retained less than about 15% of proliferation propensity compared the normal growth of the majority of clones. Besides these, other two clones showed decreased growth rate about fourfold to fivefold (Fig. 4A). The control Western blot revealed that MITF still remained gradually decreased at the time of the experiment in these clone-derived cultures maintained in DOX (Fig. 4B). Thus, some individual clones can indeed react to the lowered MITF by exclusive severe growth retardation. We hypothesize that this may happen by the absence of sufficient antiapoptotic signals that were probably almost entirely dependent on MITF in these clones. This experiment strengthens the enormous heterogeneity at the single cell level even in the relatively homologous cell line population.

Invasiveness and migration are not affected by reduced MITF levels

Because the low proliferation of melanoma cells has been reported to be associated with increased invasivity [14, 33], we have estimated invasiveness in the proliferating whole cell populations and in slowly proliferating clones. The collagen matrix invasion assay showed no significant changes between DOX-treated and non-treated cells in all cell lines (Fig. 5A) and clones (not shown). Similarly, the migration assay after cell scratches did not reveal any changes (Figs 5B and S5). Not unexpectedly, the very slow proliferation of the three clones (Fig. 4A) was accompanied with no increase in migration properties, as exemplified by the scratch assay in two clones (Fig. 5B). Next, the viability was tested in whole cell populations, and significant decrease was revealed in three cell lines (SK-MEL-3, SK-MEL-5 and SK-MEL-28) at high DOX concentrations (Fig. S6). This was in accord with the observation that these lines also revealed relatively higher growth retardation (Fig. 1D). These data indicate that viability was a sensitive assay for the detection of phenotype changes after lowering MITF and possibly reflects higher apoptosis in lower viable cells.

Reduction in MITF levels decreases expression of downstream MITF differentiation markers

MITF transcriptionally up-regulates dozens of downstream genes. Many of them are associated with the formation of the pigment

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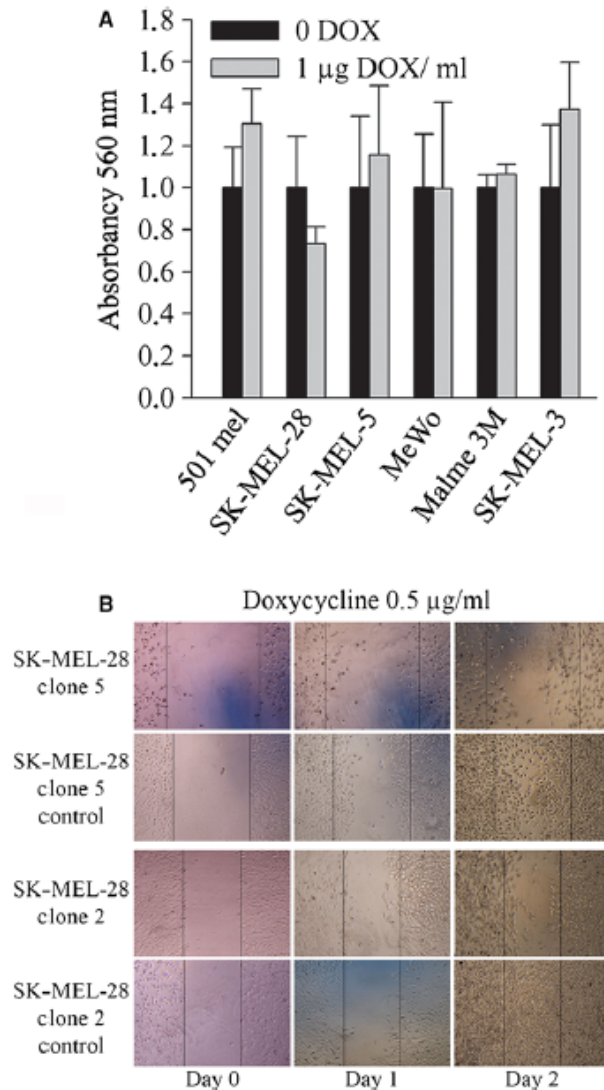
Fig. 5 Cell invasiveness of cell lines and migration assay of selected clones. (**A**) Collagen invasivity assay of six cell lines in medium without DOX and with the highest DOX concentration 1 µg/ml, performed after 6 days in appropriate medium. All results show insignificant changes in invasiveness. Two experiments with similar results were performed and one is presented. (**B**) Wound-healing assays of the two slowly proliferating clones. Note that migration in 0.5 µg/ml DOX is presented as concentrations 100 and 1000 nM produced similar results (not shown).

melanin [35]. Because melastatin, a MITF transcriptional target and a putative tumour suppressor, is sharply responding to MITF levels in melanocytes [36], we used real-time PCR to estimate the mRNA levels of melastatin, together with determining the mRNA levels of the *bona fide* MITF target tyrosinase. Maintaining cells for only 4 days in DOX caused profound decreases in melastatin and tyrosinase in 5 cell lines, while only in SK-MEL-3 cells the changes were less pronounced but significant

(Fig. 6A and B); this was possibly because the final MITF decrease was less dramatic compared to controls without DOX (Fig. 1B) in these cells. The antiapoptotic downstream MITF target livin has been also uniformly decreased in all cell lines, by Western blot (Fig. 3A). Together, differentiation has been reliably and quickly repressed by DOX-dependent down-regulation of MITF levels.

Discussion

Epithelial to mesenchymal transition is a key process associated with the invasive and metastatic disease in epithelial cancers, and EMT-like changes appear also during the phenotype switching in melanoma. Many genes change their expression during EMT-like process. The most characteristic is the down-regulation of E-cadherin and up-regulation of N-cadherin, together with the activation of SNAIL (SNAI1) and ZEB1 expression. EMT-like gene pattern has been induced in normal melanocytes by ectopic mutated BRAF [31]. Several authors have reported that melanoma has slightly atypical profile of protein expression in EMT, as SLUG (SNAI2) and ZEB2 have been presented as pro-differentiative genes coexpressed with high MITF, not participating in the EMT process [6, 29, 30, 37]. The expression of EMT markers has been found to be highly heterogeneous with the predominant EMT signature being high-N-cadherin/high-Axl/low-MITF, whereas the differentiation pattern was characterized mostly by high-E-cadherin/high-MITF in primary melanoma cell lines [38]. Even individual cells in tumours have shown different expression patterns of EMT proteins with SLUG expression weakening during tumour progression [39]. Our present results showed only two EMT-specific changes, each in other cell line (Fig. 3B), whereas the presence or changes in other EMT markers were inconsistent after the reduction in MITF expression.

We also observed no change in expression of three SC markers. Only SOX2, the expression of which was shown to require the Hedgehog signalling in melanoma and is crucial for the self-renewal and tumorigenicity of human melanoma-initiating cells [34], sharply increased in three cell lines with induced low MITF. In the remaining three cell lines, SOX2 was not expressed (Fig. 3C). Although OCT4 was found earlier increased in siRNA-MITF-transfected SK-MEL-28 cells [33], we did not observe any OCT4 changes (Fig. 3C). This discrepancy might be explained by possible more efficient reduction in MITF using siRNA-MITF. Formally, as MITF undergoes post-translational modifications that might have modulated the effect caused by MITF decrease.

Intriguingly, the highly pro-oncogenic and invasive Wnt/β-signalling pathway has been found to be anti-invasive in melanoma as β-catenin blocks invasiveness [40]. β-catenin pathway acts upstream of MITF and activates its transcription, and high-MITF levels are anti-invasive. MITF also suppresses the Rho-GTPase-regulated invading and interferes with β-catenin-induced expression of the pro-invasive enzyme membrane type 1 matrix metalloproteinase [40].

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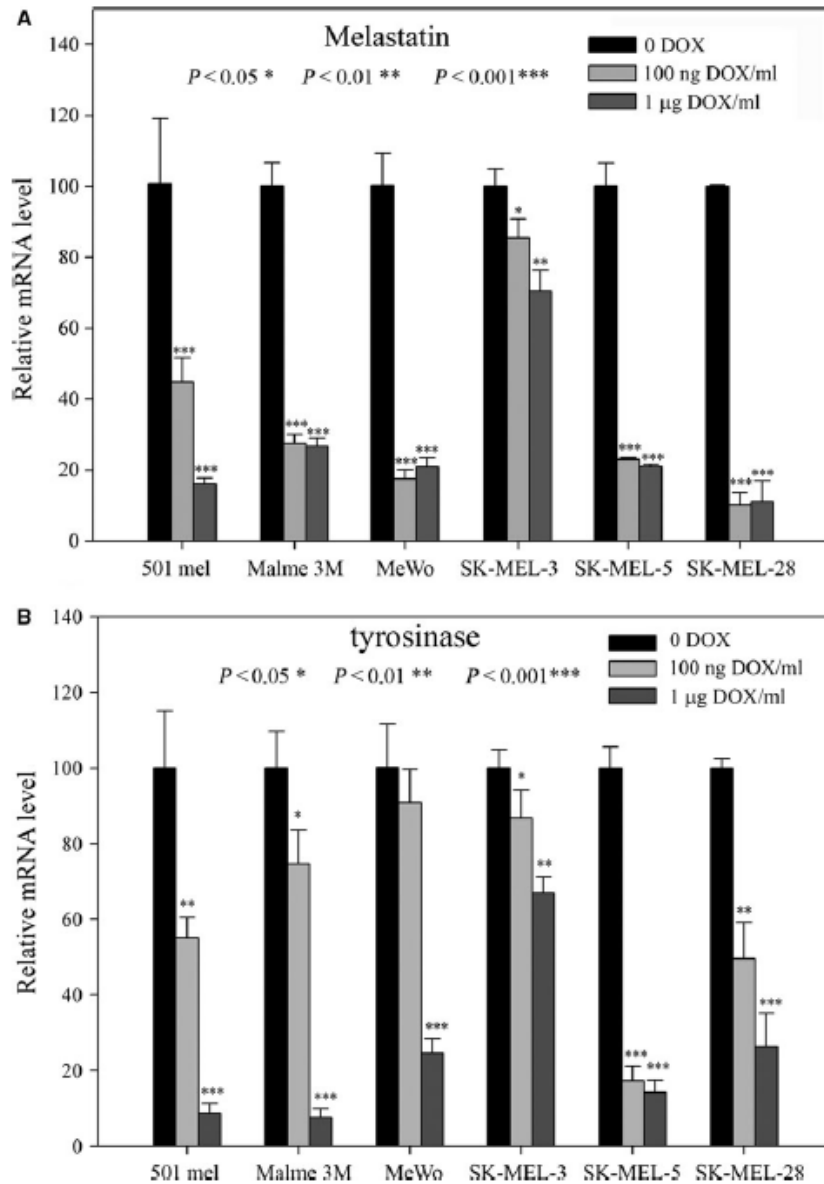
Fig. 6 Real-time PCR results detecting mRNA levels of MITF downstream differentiation markers. (**A**) The changes in melastatin mRNA levels after incubation of cells in DOX. (**B**) Levels of tyrosinase mRNA. All deviations from the −DOX controls (100 relative units) were statistically significant (with only one exception: tyrosinase in lower DOX concentration in one cell line), as depicted directly in the Figure. With the exception of SK-MEL-3 cells where the changes were small, strong decrease in RNA levels for these differentiation markers was observed.

Recently, two interesting studies which would at least partly explain the exclusive role of MITF in lowering invasiveness have implicated the expression of guanosine monophosphate reductase (GMPR), an enzyme of guanylate metabolism, in the regulation of invasiveness in melanoma cells. GMPR can deplete cellular GTP level, an event linked to lower melanoma invasiveness. The morphology of MITF-depleted invasive cells is accompanied by a larger number of invadopodia [41]. Subsequently, it has been shown that MITF is an upstream regulator of GMPR [42]. Due to the lower GTP levels in cells overexpressing MITF or GMPR, the invasiveness would be suppressed. Oppositely, when siRNA-mediated decrease in MITF was induced, with consequent declined levels of GMPR, even small increase in GTP (several per cents) generated high increase in invasion, which was even eightfold in 501mel cells and about twofold to threefold in SK-MEL-28 cells [42]. High MITF concomitantly suppressed activity of RAC1, a kinase mutated in a subset of melanomas [43], and suppression of RAC1 activity was required to reduce invasiveness. Although we have also used clones from SK-MEL-28 and SK-MEL-5 cells displaying slower proliferation, no change in invasivity was recorded. Recently, glutamine depletion was shown to be sufficient to engender the decrease in MITF and invasiveness in melanoma cells. However, the MITF decrease could not be a cause of invasiveness, as glutamine starvation led to invasivity also in MITF-negative cells [44]. The transcription factor ATF4 alone down-regulated MITF but surprisingly did not induce

invasiveness. The authors suggested a mechanism of translation reprogramming whereby the eIF2B factor was found to be a crucial driver of melanoma invasiveness. Salubrinal, which inhibits dephosphorylation of complexes acting on p-eIF2α, increased ATF4 and decreased MITF expression and induced invasiveness. Thus, as phosphorylated eIF2α inhibits eIF2B, this global reprogramming of translation involving high expression of ATF4 leads to invasiveness in melanoma cells [44]. These findings where other factors besides sole MITF decrease are required to induce invasiveness are in conformity with the findings shown here.

The antiapoptotic role of MITF in melanomas is clearly established. However, some concern remains how the antiapoptotic signals are sustained in melanoma cell lines in which MITF expression is very low or in low-MITF (and more invasive) areas of tumours. First, apparently, highly different cell context may exist among tumour cell subpopulations, and possibly single cells, that ensure antiapoptosis within the low-MITF cells. Second, another one or more antiapoptotic genes, such as Axl or others ensure that low-MITF cells do not undergo apoptosis. Previously, we have discussed whether so-called 'MITF-negative' melanomas are still melanomas, as they must have lost all MITF downstream differentiation markers [45]. We argue that such cells either die due to the lack of MITF antiapoptotic function, as already documented in 501mel cells [27], or continue growing as an undifferentiated tumour if antiapoptosis is provided by other genes. The observed low-MITF/high-Axl populations in sections of human tumours [22] could serve as a possible example. What would be also conceivable is that in the course of cell line or tumour growth, cells might have adjusted MITF levels to amounts sufficient to promote proliferation, possibly with help of other pro-proliferative (and antiapoptosis) protein(s), a notion that would reconcile both the rheostat model and our results as discussion above.

Inducibly and gradually decreased MITF level in melanoma cell lines, as described here, incurred slightly diminished proliferation, but the decrease was much smaller than anticipated taking into account the previous results [14, 18, 33]. Low-MITF populations such as some cell lines or slightly pigmented areas of tumours presumably utilize other proteins to maintain proliferation. We have observed various proliferation levels among isolated cell clones from the same cell line, indicating that even single cells in a relatively homogenous original cell line population may gradually create quite different proliferation potential when cultured longer under low-MITF conditions; the growth of small number of clones almost halted proliferation while other clones proliferated at an unchanged rate (Fig. 4A and not shown). Consistent increase in the p27 protein, although slight, seems to be a more general hallmark of MITF down-regulation. It is questionable whether the increase in p27 protein alone can incur the deceleration of proliferation in all types of low-MITF cell lines and tumours subpopulations. Predictably, p27 protein might contribute to slow proliferation in some situations *in vitro* or *in vivo*. The *in vivo* effect of p27 has not been studied extensively.

The presented results bring more complexity to the phenotype switching process with the emphasis on the cell context and individual levels of MITF in cell lines and possibly even in single cells.

It is highly probable that the primary functions of MITF in melanoma are to maintain the lineage identity (by regulating the downstream differentiation markers) and to play the indisputable antiapoptotic role. We further suggest that diminution of MITF level may accompany rather than induce the invasive phenotype in tumours, and its lower level *in vivo* may then eventually participate in the slow proliferation of the invasive tumour subpopulations.

Acknowledgements

Author contributions

J.V. and K.V. designed the study, cultured cells and carried out experiments; J.R. performed Western blots and most of other experiments; P.H. performed real-time PCR and invasivity assays; K.V. and L.O. prepared the Figures and performed statistical analysis; and J.V. wrote the manuscript. All authors have read and approved the manuscript.

Conflict of interest

The authors confirm that there is no conflict of interests.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1 Complete blocking of MITF expression achieved by transfection of shRNA-MITF cloned in pSUPER-puro plasmid followed by a short 2 days puromycin selection.

Fig. S2 Immunofluorescence with the anti-MITF antibody confirming the knockdown of MITF.

Fig. S3 Cell cycle profiles of cell lines grown with or without DOX.

Fig. S4 Proliferation of long-term cultures of cell lines in media with or without DOX.

Fig. S5 Migration (wound-healing assay) of six cell lines in – DOX and + DOX.

Fig. S6 Viability of cell lines performed in the media with indicated concentrations of DOX.

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Supporting information

Supplementary Figure Legends.

Fig. S1 Complete blocking of MITF expression achieved by a non-inducible transfection of shRNA-MITF used in this work. The target sequence was cloned in pSUPER-puroplasmid, transfected in 501mel cells,followed by a short 2 days puromycin selection. The RIPA extracts were prepared and Western blots performed. Control (scrambled) sequence did not have any effect on the MITF level. Actin has been used as a loading control confirming equal loading and the integrity of both samples.

Fig. S2 Immunofluorescence with the anti-MITF antibody confirming the knockdown of MITF. Immunofluorescence was performed with anti-MITF antibody (antibody dilution 1:200, left blocks) and DAPI (right blocks) in the identical image fields. Cells were left without DOX (upper blocks) or in 1 µg/ml DOX (lower blocks) for one week, replated into IF chambers and processed for IF next day. Scale bar 25 µm. Control cells (only two cell lines are shown as controls, similar results were obtained with the remaining four controls) do not show any difference when – DOX and + DOX fields are compared.

Fig. S3 Cell cycle profiles of cell lines grown with or without DOX. Cells were maintained in -DOX or + DOX media for one week and FACS profiles were then taken after staining DNA with propidium iodide. No appreciable changes were seen when – and + DOX cells were compared. Only in+ DOX SK-MEL-28 cells the G2/S phase peak was (paradoxically) increased.

Fig. S4 Proliferation of long-term cultures of cell lines in media with or without DOX.**(A)**Whole cell populations including control cells (containing scrambled shRNA) were cultured for five weeks in the indicated DOX concentration and then the proliferation rate experiment was performed in 24-well plates in triplicates. All cell lines grew normally during the five week period.**(B)** Control Western blot indicating the decrease of MITF was done before the experiment.

Fig. S5 Migration (wound healing assay) of six cell lines in – DOX and + DOX. The migration of six cell lines was determined by thewound healing assay as described in Materials and Methods. No differences were observed between –DOX and + DOX cultures.
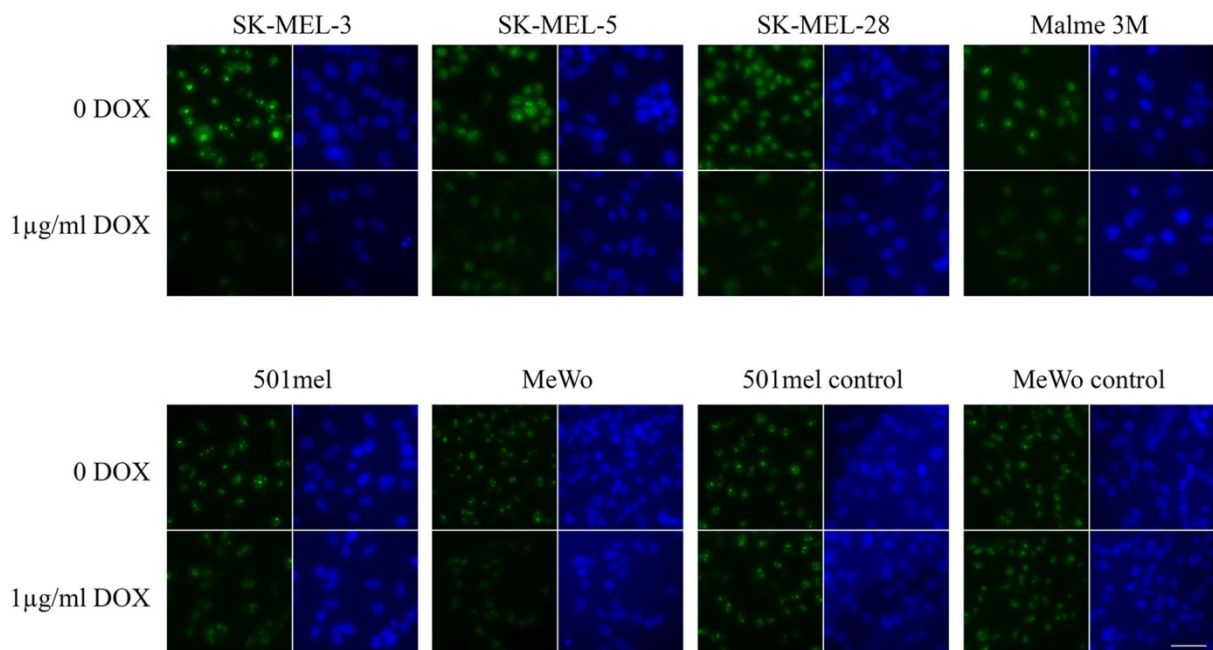
Fig. S6 Viability of cell lines performed in the media with indicated concentrations of DOX. All cell populations (including controls) were cultured for 6 days in media without or with the indicated concentration of DOX. Next day, the viability assay was performed.Cells more sensitive to MITF decrease seem to be SK-MEL-3, SK-MEL-5, and SK-MEL-28. It is a result which roughly corresponds to the slightly lowered proliferation in these cells (see Fig. 1). Predictably, lowered viability was caused by increased apoptosis in cells requiring higher MITF for proliferation.

control shRNA anti-MITF shRNA

MITF

β-actin

501mel
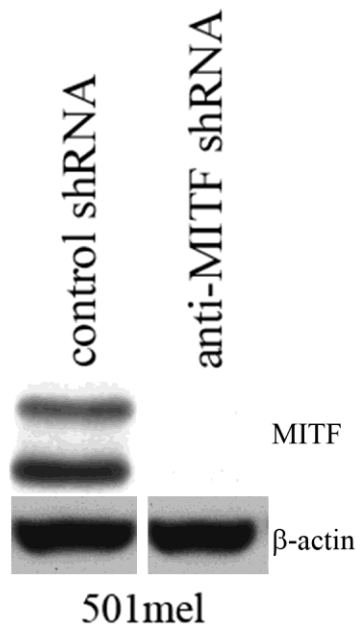
Vlčková et al.:   FIGURE S1

Fig. S1 Complete blocking of MITF expression achieved by transfection of shRNA-MITF cloned in pSUPER-puro plasmid followed by a short 2 days puromycin selection.
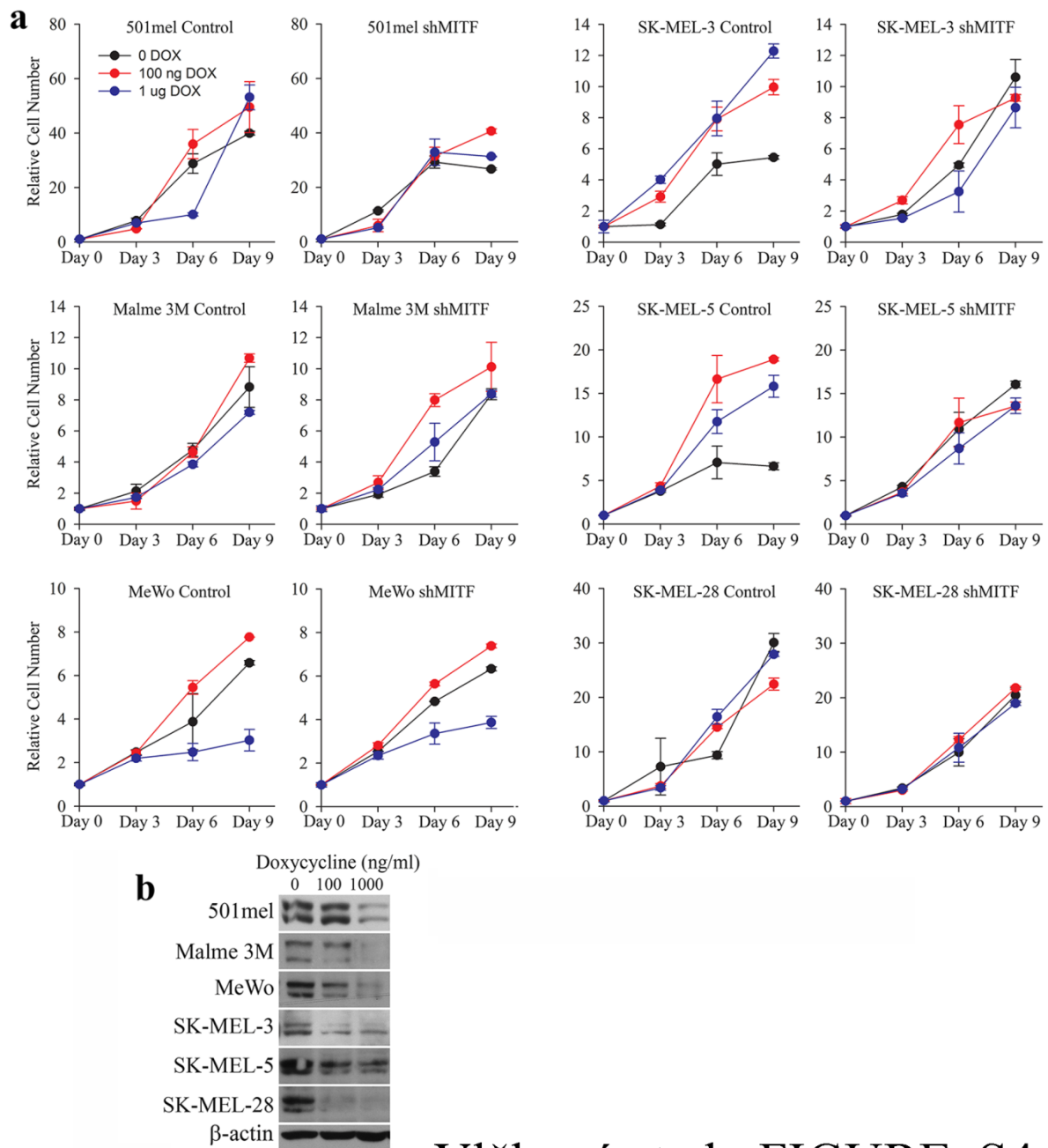

SK-MEL-3   SK-MEL-5   SK-MEL-28   Malme 3M

0 DOX

1μg/ml DOX

501mel   MeWo   501mel control   MeWo control

0 DOX

1μg/ml DOX

Vlčková et al: FIGURE S2

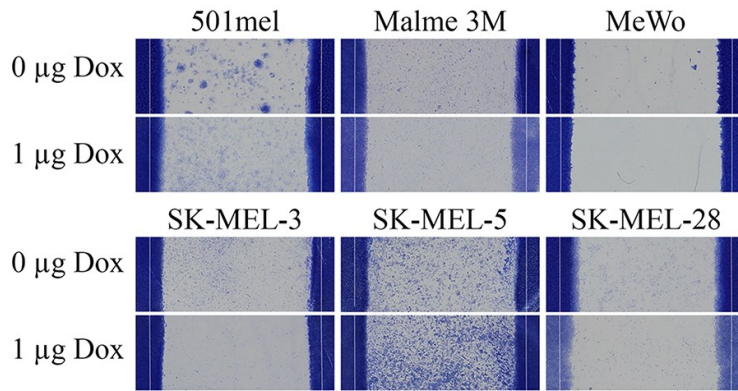**Fig. S2** Immunofluorescence with the anti-MITF antibody confirming the knockdown of MITF.
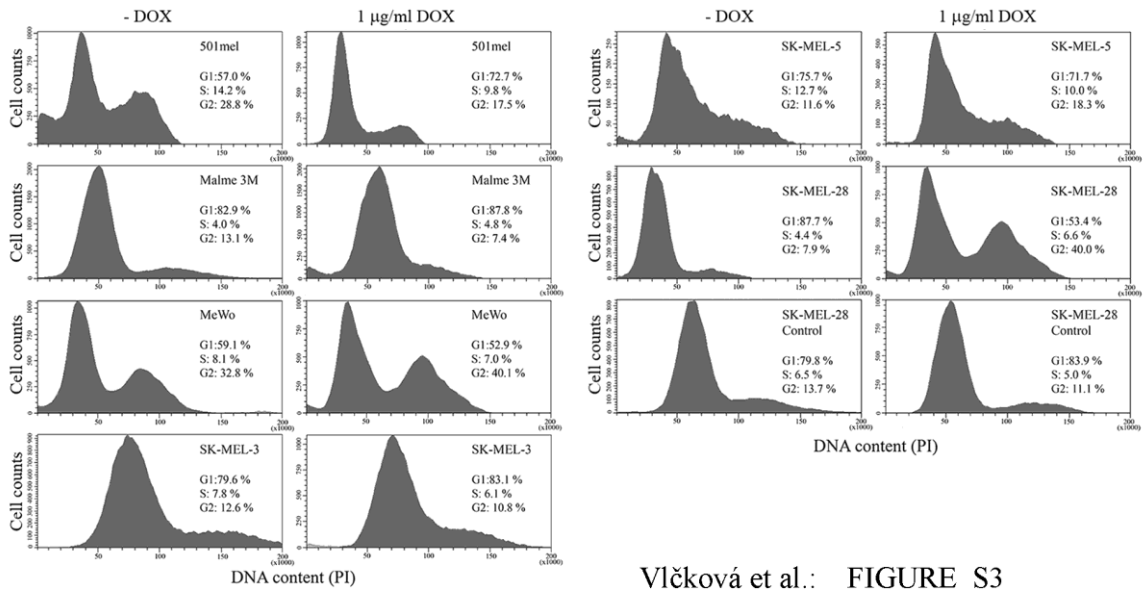
81

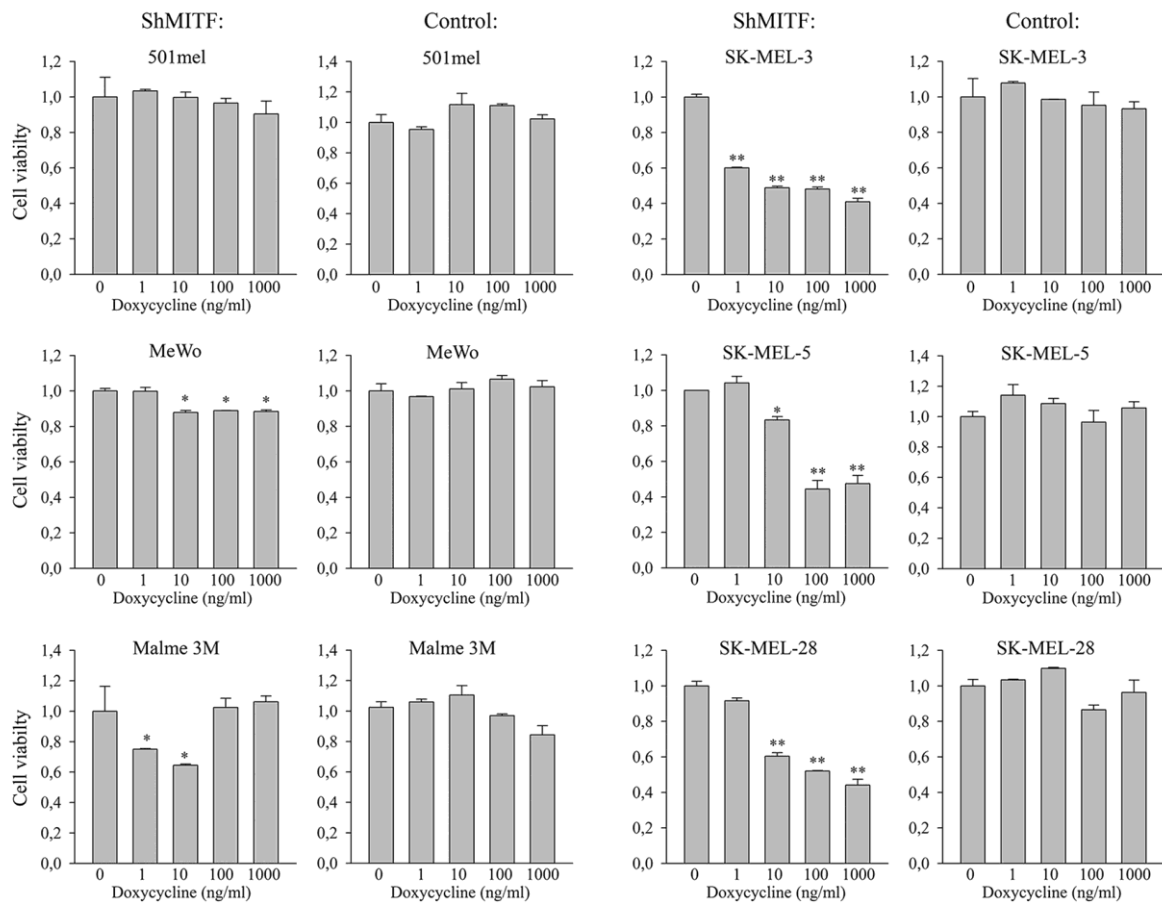**Fig. S3** Cell cycle profiles of cell lines grown with or without DOX.

Vlčková et al. FIGURE S4

Fig. S4 Proliferation of long-term cultures of cell lines in media with or without DOX.

Vlčková et al: FIGURE S5

Fig. S5 Migration (wound-healing assay) of six cell lines in – DOX and + DOX.

Vlčková et al.: FIGURE S6

Fig. S6 Viability of cell lines performed in the media with indicated concentrations of DOX.

4.5 RESULTS AND DISCUSSION

In the submitted doctoral thesis, I focus on the role of the HH signaling in tumorigenesis. I present new findings of HH target genes, the activity of HH pathway in tumor cells, targeting the HH pathway and discuss the role of HH pathway in melanoma cell lines. HH signaling is deregulated in various tumors and affects different cellular processes such as apoptosis, proliferation, epithelial-to-mesenchymal transition, invasion and metastasis. Our research group is focused on melanoma research. Thus, the main experiments were performed on melanoma cell lines and supplemented by cell lines of various tumor origins.

In the first paper **"Survivin, a novel target of the Hedgehog/GLI signaling pathway in human tumor cells."** We newly demonstrated that the anti-apoptotic protein survivin (also called BIRC5) is a direct target of the HH signaling pathway. Survivin is a member of the inhibitors of apoptosis (IAP) protein family. IAPs have an important role in the regulation of programmed cell death, response to stress signals or genomic instability. Survivin is overexpressed in virtually all human cancers and is associated with a poor prognosis of patients. The precise mechanism of how survivin affects apoptosis is not known. It was suggested that survivin could directly block caspase-3 and caspase-9. It was also proposed that survivin together with XIAP (another member of IAPs) inhibit caspase-9. Moreover, survivin blocks pro-apoptotic protein SMAC/DIABLO (second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein) and thus inhibits intrinsic apoptotic pathway (Garg et al., 2016).

The findings in the regulation of survivin expression are crucial for further cancer research because survivin is aberrantly activated in the vast majority of tumors. It was shown that the survivin promoter contains binding sites of pro-oncogenic transcription factors such as SP1, STAT3, NF-κB, E2F1, KLF5, or DEC1 (Boidot et al., 2014). These transcription factors are linked to malignant characteristics of tumors. SP1 (Specific protein 1) expression is associated with poor prognosis of cancer patients and plays many roles in cancers (Beishline and Azizkhan-Clifford, 2015). It was suggested that SP1, together with SP3 (specific protein 3), regulates the basal transcription of the *survivin* gene (Xu et al., 2007). KLF5 (Kruppel-like factor 5) for example increases proliferation and metastasis in thyroid carcinoma (Ma et al., 2018) or is associated with a poor prognosis of patients with pancreatic

cancer (Li et al., 2019b). NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling plays many important roles in cancer (Xia et al., 2014).

In this publication, we show that GLI transcription factors are involved in the regulation of the *survivin* gene and propose that *survivin* is the direct target of the Hedgehog signaling pathway. We found 11 potential GLI binding sites in *survivin* promoter (4.1, Fig.1, p.32). However, none of them had the consensus sequence GACCACCCA defined by Kinzler and Vogelstein (1990). GLI binding sites in the *survivin* promoter had from one to three mismatches. It was shown that binding sites containing more than one substitution (mismatch) can be active. GLI binding sites with two or more substitutions were found in promoters of many known GLI target genes (Winklmayr et al., 2010). We determined that no single binding site exhibits the capability to activate the *survivin* promoter. However, GLI binding site occupying +2 to +10 area of the promoter (relative to the start of translation) seems to have an inhibitory character in reporter assays (it might have different characteristics in genomic context, but it was not studied so far) (4.1, Fig.2, p.33). Similarly, we did not find the specific combination of GLI binding sites crucial for survivin expression.

Next, we observed that the activity of the survivin promoter is decreased after cyclopamine (inhibitor of SMO) or GANT61 (inhibitor of GLI1 and GLI2) treatment in promoter-reporter assays. GLI1 and GLI2 increased the activity of the promoter, GLI2 elevated the activity more than GLI1 (4.1, Fig.3, p.34).

Moreover, GANT61 treatment affected the endogenous survivin mRNA and protein levels in a majority of cell lines from a large panel of 40 cancer cell lines of various origins (4.1, Suppl.TableS1, p.42). The survivin protein levels decreased massively in few cell lines, such as melanoma cell lines SK-MEL-3 and WM35 or adrenocortical carcinoma cell line SW-13 after 24h treatment of 10 µM or 20 µM GANT61 (4.1, Fig.4, p.35). However, a portion of cell lines showed any or only minimal decrease of survivin expression at these GANT61 concentrations (see 4.1, Suppl.Fig.S3, p.44). Similarly, real-time PCR revealed that mRNA levels more or less responded to the GANT61 treatment, the mRNA level decreased in most of cell lines. We were not able to elucidate why the survivin levels did not decrease in some cell lines. In our next publication (Réda et al., 2018), we showed that the main components of the canonical HH signaling pathway were expressed in the majority of tested cell lines. Thus, we propose that higher concentrations of GANT61 could be used to achieve a more substantial decrease in GANT61 non-responding cell lines. We also suggest that other transcription factors play a crucial role in maintaining basal survivin expression levels in cell

lines that do not respond to GANT61 treatment. For example, it was shown that the SP1 transcription factor regulates survivin expression in cell line A549 (Chen et al., 2011).

In western blot analysis we also examined the expression of anti-apoptotic protein BCL2. *BCL2* gene was described as a direct target gene of the HH pathway effectors – GLI2 (Regl et al., 2004) or GLI1 (Bigelow et al., 2004). The expression of BCL2 decreased after GANT61 treatment only in melanoma cell line SK-MEL-28, in the rest of cell lines the BCL2 protein level did not altered. Other research groups also reported that BCL2 did not respond to GANT61 treatment. Any decrease of BCL2 was observed after GANT61 treatment in cell lines of hepatocellular carcinoma (Wang et al., 2013). Lin et al. (2016) showed only a slight decrease of BCL2 protein in medulloblastoma cells after 40 µM GANT61 treatment. Thus, we proposed that the GLI transcription factors could affect the BCL2 expression in specific cell types or other transcription factors are able to maintain the expression of BCL2 after inhibition of GLI factors.

We showed that GLI2 bound to the endogenous survivin promoter and affected endogenous level of survivin protein. We performed chromatin immunoprecipitation and proved that ectopic ΔNGLI2 (which is commonly used because it lacks the N-terminal suppressor domain) directly bound to the endogenous survivin promoter in A549 cells (4.1, Fig.5, p.36). Moreover, ectopic expression of ΔNGLI2 was able to increase the expression of endogenous survivin in human fibroblast IMR-90 (4.1, Fig.5, p.36). We were not able to detect the endogenous level of survivin in IMR-90, although Huang et al. (2009) were able to visualize the basal level of survivin in IMR-90. The discordance can be caused by using a different antibody against survivin.

We also observed the overlap of GLI2 and survivin expression in immunohistochemically stained tumor sections (4.1, Fig.6, p.37). Similarly, we showed co-localization of GLI2 and survivin protein in tumor sections under immunofluorescence microscopy (4.1, Fig.6, p.37).

Moreover, we demonstrated that GANT61 partially decreased tumor growth and lowered survivin expression in vivo in GANT61-sensitive melanoma cell xenografts (4.1, Suppl.Fig.S7, p.45)

We conclude that survivin is a direct target gene of the HH signaling pathway. It is consistent with a study performed by Brun et al. (2015), who showed that survivin is overexpressed in HH-driven medulloblastoma. Another study demonstrated a positive correlation between survivin and GLI2 in ovarian carcinoma samples (Ozretić et al., 2017). Even though we showed that GLI2 regulates survivin expression, its expression was not

lowered after GANT61 treatment in some cell lines. It implicates an important role of the other transcription factors in the regulation of the survivin expression or a requirement of higher GANT61 concentrations for the repression of survivin level. Moreover, it was also described that survivin expression is regulated epigenetically. Our group previously showed that survivin is regulated by SWI/SNF chromatin remodeling complex in melanoma cells (Ondrušová et al., 2013). The epigenetic regulation by BMI1, a key component of Polycomb repressive complex1 was also described (Acquati et al., 2013).

Our results highlight the role of the HH signaling pathway in the regulation of apoptosis. Anti-apoptotic protein XIAP was previously described as a direct target of the HH pathway (Kurita et al., 2011). It was also described that survivin together with XIAP inhibits caspase-9 (Garg et al., 2016). It implies that this XIAP/survivin anti-apoptotic pathway could be under HH-signaling control. Moreover, Meister et al. (2018) showed that HH signaling decrease the level of pro-apoptotic protein Noxa. Thus it seems that the HH signaling pathway has a crucial role in anti-apoptotic signaling. It should be investigated whether the HH pathway affects the expression of other pro-apoptotic and anti-apoptotic proteins. The precise role of HH signaling in apoptosis should be elucidated.

Moreover, our findings can be important for the following research, for example in the targeted therapy. Combination therapy targeting HH signaling pathway and survivin can be very beneficial for cancer patients.

We aimed to find a potent combination of GANT61 and an inhibitor of another important signaling pathway in our following study **"GLI inhibitor GANT61 kills melanoma cells and acts in synergy with obatoclax".**

The treatment of melanoma with MEK (Mitogen-activated protein kinase kinase) kinase inhibitors or kinase inhibitors directed against mutated BRAF(V6000E) seemed to be very promising, but acquired resistance invariably appeared after a few months of treatment (Davies and Kopetz, 2013; Kozar et al., 2019). In the recent decade, the role of HH signaling in melanoma has been elucidated. It was shown that GLI2 is inversely correlated with MITF, a crucial factor in melanoma transcription circuitry (Javelaud et al., 2011). High GLI2 expression and low MITF expression are connected with the highly invasive phenotype of melanoma cells (Alexaki et al., 2010; Javelaud et al., 2011; Pierrat et al., 2012). In our previous publication, we showed that GANT61 inhibits survivin, a target gene of GLI transcription factors, in many cancer cell lines including few melanoma cell lines (Vlčková et al, 2016a). Using GANT61 is beneficial because it blocks GLI factors also in case they are

activated non-canonically. Therefore we used GANT61 alone or in combination with obatoclax (inhibitor of BCL2 family proteins) for melanoma cells treatment in the presented study.

At first, we confirmed that GLI2 is expressed in all tested melanoma cell lines by Western blots analysis. We detected the GLI2 expression in the 501mel cell line that was previously described as GLI2 negative (Javelaud et al., 2011). We showed that MITF and GLI2 are inversely correlated in most cell lines as described by Javelaud et al. (2011) (4.2, Fig.1, p.48). We observed that GANT61 decreased the activity of potent GLI2 reporter construct in promoter-reporter assays (4.2, Fig.1, p.48).

Next, we tested whether GANT61 affects the expression of MITF because it was previously described that GLI2 represses transcription of MITF (Pierrat et al., 2012). We expected the elevation of MITF expression after the GANT61 treatment. However, no increase of MITF expression was observed in any of the four tested melanoma cell lines. Instead, MITF decreased in SK-MEL-5 treated with 10 µM GANT61 and after 20 µM GANT61 treatment the MITF expression decreased massively. The decrease of MITF expression was also observed in MeWo and SK-MEL-28. In 501mel cell line, the expression of MITF lowered very slightly (4.2, Fig.2, p.49). These results were quite surprising because they do not reflect the consensual GLI2/MITF inversion model proposed by Javelaud et al. (2011). The model of high-MITF/low-GLI2 was also confirmed by Faião-Flores et al. (2017), who showed that GLI2 and MITF are inversely correlated in vemurafenib-resistant melanoma cells (vemurafenib is kinase inhibitor directed against mutated BRAF(V6000E). In that study, GANT61 treatment decreased *GLI2* mRNA level and increased *MITF* mRNA level. The authors also investigated the relationship between GLI2 and MITF protein levels in vivo by examining the expression of both proteins in The Cancer Genome Atlas melanoma cohort. These data also proved the inverse correlation between GLI2 and MITF. Thus we can only discuss why MITF does not respond to GANT61 treatment in our experiment. We propose that GLI2 may not be the repressor of MITF transcription in every cell context. We also suggest that GLI2 can possibly regulate the MITF level indirectly.

Next, we demonstrated that melanoma cells incubated for 72-h with 25 µM GANT61 had strongly decreased viability. Control non-melanoma cancer cell lines had also lowered viability after GANT61 treatment, only A549 cell line, originated from lung carcinoma, was GANT61 resistant (4.2, Fig.3, p.50). The most GANT61 sensitive cells SK-MEL-3 and BEU were used for soft agar assay. GANT61 blocked the colony formation in both cell lines, although some colonies were observed in BEU cell line. The combination of GANT61 and

obatoclax completely inhibited colony formation in both cell lines (4.2, Fig.3, p.50), implicating that GANT61+obatoclax treatment could be used as a potentially beneficial combination in other experiments.

Even though it was well described that GANT61 induces apoptosis in various cancer cells (Lin et al., 2016; Matsumoto et al., 2014; Tong et al., 2018), we examined whether it does so in melanoma cell lines as well. Flow cytometry analysis after GANT61 treatment confirmed massive apoptosis in GANT61 treated cells. We also observed nuclei fragmentation and chromatin condensation indicating apoptosis (4.2, Fig.3, p.50). Thus we proved that GANT61 causes apoptosis in melanoma cells.

At last, we were looking for an efficient combination of drugs decreasing the survival of melanoma cells. We combined 20 µM GANT61 with inhibitors of various signaling pathways. We studied combination with 300nM selumetinib (MEK inhibitor), 300nM AZD5363 (AKT kinase inhibitor) and 100 nM obatoclax (BCL2 protein family inhibitor). Neither selumetinib nor AZD5363 improved the effect that had GANT61 alone. Obatoclax alone had no effect on melanoma cells but enhanced the effect of GANT61 treatment in 6 tested melanoma cell lines (501mel, Hbl, SK-MEL-28, MeWo, WM1552C, SK-MEL-5). In three other cell lines, no improvement was observed after adding obatoclax to GANT61 (see 4.2, Fig.4, p.51). Calculation of combination index revealed the strong synergistic effect of obatoclax and GANT61 (4.2. Table II, p.51).

Our finding of a novel potent combination of anticancerous drugs that dramatically decreases proliferation of melanoma cells is important from the point of view that monotherapies are often followed by acquired resistance. The benefit of using inhibitor obatoclax was showen by Haq et al. (2013). They determined that deregulated expression of proteins from the anti-apoptotic BCL2 family plays a crucial role in melanoma resistance to apoptosis. They observed that combination treatment with obatoclax and BRAF inhibitors overcomes the resistance to BRAF inhibitors. Generally, the treatment with combined therapy is believed to be more efficient and to have a better long-term effect than monotherapies. Few combination therapies of melanoma are in clinical trials. Recently the results from phase Ib clinical trial NCT02130466 using triple-combined therapy of dabrafenib (inhibitor of mutated BRAF), trametinib (MEK kinase inhibitor) and pembrolizumab (antibody against programmed death1) in melanoma patients were published. This combination therapy shows a high initial response rate and the response lasts approximately one year. A subset of patients showed the response with a duration of approximately 2 years without acquired resistance (Ribas et al., 2019).

"Widespread Expression of Hedgehog Pathway Components in a Large Panel of Human Tumor Cells and Inhibition of Tumor Growth by GANT61: Implications for Cancer Therapy." is our next publication. We examined the expression of individual components of the HH signaling pathway in 56 cell lines of various origin (53 cancer cell lines, 3 control non-cancerous cell lines) by Western blot analysis (4.3, Fig.1, p.56). We examined the expression of proteins SHH, PTCH1, SMO, SUFU, GLI1, GLI2 and GLI3. In general, we determined that all mentioned proteins are expressed in the vast majority of cell lines. The effectors of the HH pathway were expressed in all cell lines. It is not so surprising because mounting evidence shows that GLI factors can be activated even though the upstream canonical HH pathway is not active (Pietrobono et al, 2019). It was more interesting that SHH protein was expressed in all cell lines, although in some of them weakly. It implicated that the HH signaling pathway was canonically activated by SHH in all cell lines in an autocrine or paracrine manner. We also examined the expression of survivin and BCL2 that are direct target genes of the HH pathway. Survivin was expressed in all cancer cell lines, but we were not able to detect its expression in control cell lines IMR-90 and WI-38 (human fibroblasts). BCL2 was expressed in 41 cell lines, including control cell lines.

Next we studied how treatment with GANT61 affects various cell lines. In proliferation assay, GANT61 treatment completely eradicated cells of melanoma, osteosarcoma, neuroblastoma and small cell lung cancer cell lines (4.3, Fig.2, p.57). Oppositely, non-small cell lung cancer cell line A549 and pancreatic cancer cell lines were GANT61 resistant. It was rather surprising that all pancreatic cell lines were GANT61 resistant because blocking of HH signaling by HH inhibitors had promising results in preclinical studies. For example, sonidegib treatment reduced tumor volume and prolonged survival in islet cell neoplasm in a mouse model (Fendrich et al., 2011). On the other hand, clinical trials of HH inhibitors in combination therapy failed as they were not beneficial against standard treatment (Katoh, 2019). The pancreatic cell line PANC-1 was previously described as cyclopamine and GANT61-resistant. However, the combination therapy of GANT61 and the inhibitor of the mTOR pathway rapamycin led to the decreased cell viability and sphere formation of PANC-1 (Miyazaki et al., 2016). The exact mechanism of GANT61 resistance needs to be elucidated.

It was previously described that GANT61 treatment induces apoptosis in various cancer cell lines (Huang et al., 2014; Matsumoto et al., 2014). We showed in our previous study (Vlčková et al., 2016, see 4.2) that GANT 61 causes apoptosis in melanoma cell lines

SK-MEL-3 and SK-MEL-5. Here, we proved this effect of GANT61 once more by TUNEL assay. We observed a massive apoptosis in GANT61 treated cells. We also detected apoptotic nuclei in GANT61 treated cells. Thus we confirmed that GANT61 induces apoptosis in melanoma cell lines. Similarly, Faião-Flores et al. (2017) has recently shown that GANT61 induced apoptosis in melanoma cells (but they used different cell lines). These observations are in accordance with the role of HH signaling in the regulation of anti-apoptotic proteins. However, in melanoma, it is believed that anti-apoptotic characteristics of tumors are maintained by MITF, a key regulator of many transcripts in melanoma. MITF positively regulates the expression of anti-apoptotic protein BCL2 and it seems that it also regulates the expression of anti-apoptotic protein BIRC7 (ML-IAP) (Goding and Arnheiter, 2019).

Our results show widespread expression of the main components of the HH signaling pathway in cell lines of various origins. We also show that GANT61 induces apoptosis in melanoma cells. It is in accordance with our previous results that the HH pathway plays an important role in maintaining the anti-apoptotic character of cancer cells (Vlčková et al., 2016a).

Our following study was focused on melanoma. MITF is a melanocytic lineage-specific transcription factor that is crucial in melanoma development and progression. It was described that the different levels of MITF expression are linked to melanoma cell growth, proliferation, survival, differentiation, invasion and metastasis. It was described as MITF "rheostat model" or "phenotype switching" (Carreira et al., 2006; Hoek and Goding, 2010). According to the model, phenotypically distinct populations of cells with different MITF levels are found in melanoma: high-MITF cells are associated with differentiation and increased proliferation but low invasion, low-MITF cells are more invasive and their proliferation is decreased. In our study **"Inducibly decreased MITF levels do not affect proliferation and phenotype switching but reduce differentiation of melanoma cells."** we established cell lines with inducibly regulated MITF levels. We aimed to characterize better the role of MITF in phenotype switching. The inducible regulation of MITF was achieved by using doxycycline (DOX)-based inducible lentiviral system (Tet-on system) in six melanoma cell lines with a high or average basal level of MITF. DOX in doses 0-1 µg/ml decreased the protein expression of MITF, albeit sometimes not completely (see 4.4., Fig.1, p.69/70). It was very surprising that the observed decrease of MITF did not cause any (or a very slight) effect on cell proliferation see (4.4, Fig.1, p.69/70). We did not see any or very slight reduction of proliferation rate in a long-term cultivation in DOX (4.4, Suppl.Fig.S4, p.83). Moreover, we

expanded 15 clones from SK-MEL5 and SK-MEL-28 cell lines and maintained them in DOX media (1 μg/ml max). We observed the reduced proliferation in one-third of clones (4.4, Fig.4, p.73). The proliferation of two clones decreased by 15% related to control and in the remaining three clones the proliferation was reduced four to five folds. These results indicate single-cell heterogeneity in established "homologous" cell line population. These results differed from the previous studies, in which high-MITF level was described as a pro-proliferative factor and the low-MITF level was linked to decreased proliferation. (Carreira et al, 2006; Hoek and Goding, 2010;Goding and Arnheiter, 2019). Therefore we decided to examine the proliferation rate and MITF levels of native melanoma cell lines. We determined that the proliferation rate of six melanoma cell lines did not correlate with MITF expression level. For example, DOR cell line proliferated the most rapidly, although DOR had the almost lowest expression of MITF from all tested cell lines (4.4, Fig.2, p.71). These results do not correlate with the rheostat model and imply that no specific basal level of MITF is connected to high or low proliferation rate.

Moreover, we showed that neither invasion (4.4, Fig.5, p.74) nor migration (4.4, Suppl.Fig. S5, p.84) was affected by DOX-decreased MITF levels in melanoma cell lines These results were also not in accordance with the rheostat model as low-MITF cells should be more invasive than high-MITF cells.

Next, we tested whether the expression of downstream target genes of MITF decreased in correlation with the lowering expression of MITF (4.4, Fig.3, p.72). We observed that mRNA level of differentiation markers melastatin and tyrosinase lowered in response to decreasing level of MITF in accordance with the rheostat model. Transcriptional targets of MITF, livin and BCL2 were also examined. The expression of livin decreased in correlation to MITF level, but the expression of BCL2 did not lower (will be discussed below). Low MITF state is connected with increased levels of p27 (Carreira et al., 2006) and AXL (AXL receptor tyrosine kinase) expression (Sensi et al., 2011). The increase of p27 was observed in all investigated cell lines, whereas the increase of AXL (in response to decreased MITF) was observed in MeWo cell line, in Malme3M AXL expression did not changed; in the rest of cell lines the expression of AXL was not detected.

We were also interested, whether decreased MITF level changed the EMT-like characteristics of melanoma cells. Low MITF cells are linked with an increased invasion and invasive cells are often linked with EMT. Western blot analysis showed only minimal changes in expression levels of EMT markers E-cadherin, N-cadherin, Slug (SNAI2), Snail (SNAI1), Vimentin, ZEB1 and ZEB2. Only two noticeable changes were seen – the decrease

of E-cadherin in MeWo cells and the increase of N-cadherin in SK-MEL-28. Thus, we can say that changes in MITF expression do not affect EMT-characteristics.

Similarly, we analyzed the expression of stemness markers and showed that the level of SOX2 (sex-determining region Y- BOX2) protein increases with decreasing MITF. SOX2 is essential for the cell-renewal and tumorigenicity of melanoma-initiating cells. Interestingly, the HH signaling pathway plays an important role in the regulation of *SOX2* gene (Santini et al., 2014).

We conclude that our results are not entirely in accordance with the rheostat model. In this respect, we somewhat revised the rheostat model. We showed that decreasing levels of MITF protein do not correlate with changes in proliferation rate, invasion, migration or EMT. The pitfalls of MITF rheostat model were discussed from many points of view (Seberg et al., 2017; Vachtenheim and Ondrušová, 2015) . Wellbrock and Arozarena (2015) conclude that the expression level of MITF is not the only "characteristic" that defines the activity of MITF, although it is well measurable and changeable. They point out that MITF often acts in an opposite manner at similar expression levels but in a different context. Thus, it is clear that more research is needed to define the role of MITF in "the rheostat model" more precisely.

Our results were discussed by Goding and Arnheiter (2019) in extensive MITF review. Prof. Colin Goding, who established the rheostat model, agreed our results. We provided him our inducible cell lines for his future research.

The results from the presented rheostat model study can also be discussed from the point of view of HH signaling pathway context. In recent years, few publications appeared in which the role of HH signaling in melanoma was discussed. It was shown that GLI2 is inversely correlated with MITF (though in different cell lines than we used), a crucial factor in melanoma transcription circuitry (Javelaud et al., 2011; Faião-Flores et al., 2017). Thus, we can discuss the obtained result from the point of view that decreased expression of MITF is linked to increased expression of GLI2. Alexaki et al. (2010) performed a study similar to ours, but they changed the expression level of GLI2. They worked with 10 melanoma cell lines split according to GLI2 level as high-GLI2 and low-GLI2. They worked with different cell lines than us. The exception was 501 mel that they classified as low-GLI2 because the mRNA and protein levels were weakly detected. However, we previously observed that 501mel has relatively strong expression of GLI2 (Vlčková et al., 2016b - 4.2, Fig.1, p.48). This discordance can be caused by using a different primary antibody. Alexaki et al. determined that high-GLI2 cell lines are more invasive than low-GLI2. Moreover, they observed that the decrease of GLI2 protein in high-GLI2 cells caused reduced cell migration

and invasion. Thus, the study of Alexaki et al. indicates that there could be a negative correlation between GLI2 and MITF. Since low-GLI2 should be connected to high-MITF and low invasion. Oppositely, high-GLI2 should be linked to low-MITF and high rate of invasion. These characteristics fit well with results of Alexaki´s study. The problem of our study and that of Alexaki et al. is that MITF expression is not in GLI2 expression context and vice versa. In our previous study (4.2, Fig.1, p.48), we showed that low-GLI2/high-MITF and vice versa are roughly correlated in cell lines used in the presented study (with the exception of cell line Malme 3M that was not included to the study from 2016).

Alexaki et al. (2010) found that cell proliferation rate is not correlated with GLI2 expression level in 10 melanoma cell lines. We observed a similar result at growth curves of 6 cell lines with different MITF levels (4.4, Fig.2, p.71) Thus, it seems that no particular basal GLI2 or MITF expression level is connected to low or high proliferation rate.

When we look at other results from our study, we can discuss the Western blot analysis of various proteins (4.4, Fig.3, p.72) – targets of MITF, EMT markers, SC markers. As mentioned above, BCL2 protein expression did not decrease in response to lowering MITF expression. The anti-apoptotic BCL2 gene was described as a MITF target gene (McGill et al., 2002). Thus we could expect that BCL2 expression should correlate with the expression of MITF, but it obviously does not occur. However, BCL2 was also described to be a target of GLI2 (Regl et al., 2004). Thus we can speculate that both transcription factors MITF and GLI2 affect the expression of BCL2 in melanoma cells. Since GLI2 and MITF are negatively correlated, GLI2 could compensate the downregulated expression of MITF that could result in the continual expression of BCL2 irrespective of MITF expression level.

GLI transcription factors are also connected with the EMT markers regulation as EMT markers are GLI target genes (Katoh and Katoh, 2009; Gonzales and Medici, 2015). In the panel of EMT markers we can see only two noticeable changes in protein expression. E-cadherin decreases in MeWo cell line and N-cadherin decreases in SK-MEL-28. N-cadherin also seems to increase slightly in MeWo and Slug (SNAI2) increases slightly in SK-MEL-5. These changes in expression level of EMT markers can be possibly caused by increasing GLI2 expression level.

Low-MITF/high-AXL/high-GLI2 state is connected with low proliferation and differentiation and increased invasion. Low MITF cells also have CSC-like characteristics (Vachtenheim and Ondrušová, 2015). From this point of view, increasing SOX2 expression in MITF decreasing manner is very interesting. SOX2 is a crucial transcription factor for maintaining a self-renewal of cancer stem cells. In melanoma, SOX2 regulates the expression

of other stem cell markers as NANOG, OCT4 or KLF4 (Vachtenheim and Ondrušová, 2015). Moreover, SOX2 is regulated by GLI1 and GLI2 (Santini et al., 2014). Therefore, it seems that increasing expression of GLI2 could be the mechanism that could explain the higher expression of SOX2 in low-MITF cells. It would be in accordance with the finding that the HH pathway is activated in cancer stem cells (Cochrane et al., 2015; Takebe et al., 2015).

To conclude, it seems that some results can be explained from the point of view of low-MITF/high-GLI2 state in melanoma cells. Our hypothesis about the role of GLI2 should be investigated in the following research. Other similar studies of MITF should be performed in the GLI2 context.

5 CONCLUSIONS

Hedgehog signaling pathway plays a crucial role in embryonic development and when aberrantly activated in cancer initiation and progression. The results presented in this doctoral thesis bring new findings about the role of HH signaling and HH targeted therapy in cancer, especially in melanoma.

- We found that the anti-apoptotic protein survivin, associated with a poor prognosis of cancer patients, is regulated by the HH signaling pathway. We proved that the survivin gene is a direct target of the GLI2 transcription factor.

- We showed that the inhibitor of GLI1 and GLI2 transcription factors GANT61 can inhibit survivin in cancer cells.

- We found a new combination of anticancerous drugs that eliminate melanoma cells in vitro. We found that obatoclax, the inhibitor of the BCL2 protein family, has no effect if used alone. We found that a combination of obatoclax and GANT61 eradicates melanoma cells in few days in vitro.

- We conclude that all main components of the HH pathway are expressed in the most of cancer cell lines that we tested (56 cell lines). It implies that the canonical Hh pathway is active in cancer cell lines of various origins

- We show that GLI1 and GLI2 inhibitor GANT61 eradicates melanoma cells in vitro by the process of apoptosis.

- We established cell lines with inducibly regulated MITF level by shRNA and aimed to characterize better the role of MITF in phenotype switching. We show that proliferation, migration and invasion do not change with decreasing MITF expression level. Our results differ from the "rheostat model" proposed for MITF transcription factor in melanoma cells. We suggest that only changes in MITF expression level do not explain the changes of cellular characteristics such as proliferation or invasion.

- We observed that the expression of SOX2 increases with decreasing levels of MITF. We propose that the increase is caused by the high expression of GLI2, the effector of HH signaling pathway.

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7 APPENDIX

PERMISSIONS TO REPRINT PUBLICATIONS:

Publication I
Vlčková, K., Ondrušová, L., Vachtenheim, J., Réda, J., Dundr, P., Zadinová,M.,Žáková,P.,& Poučková, P. (2016). Survivin, a novel target of the Hedgehog/GLI signaling pathway in human tumor cells. *Cell Death and Disease*, *7*(1), e2048.

Publication III
Réda, J., Vachtenheim, J., Vlčková, K., Horák, P., & Ondrušová, L. (2018). Widespread expression of hedgehog pathway components in a large panel of human tumor cells and inhibition of tumor growth by GANT61: Implications for cancer therapy. *International Journal of Molecular Sciences*, *19*(9).

Publication IV
Vlčková, K., Vachtenheim, J., Réda, J., Horák, P., & Ondrušová, L. (2018). Inducibly decreased MITF levels do not affect proliferation and phenotype switching but reduce differentiation of melanoma cells. *Journal of Cellular and Molecular Medicine*, *22*(4), 2240–2251

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