Ligand-dependent Hedgehog pathway activation in Rhabdomyosarcoma: the oncogenic role of the ligands

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Background: Rhabdomyosarcoma (RMS) is the most common type of soft tissue sarcoma in children. RMS is thought to derive from embryonic cells committed to developing into skeletal muscle and is divided into two major subtypes in childhood: embryonal and alveolar (eRMS and aRMS, respectively). The majority of aRMS (80–85%) contain one of the reciprocal chromosomal

Rhabdomyosarcoma (RMS) is the most common type of soft tissue sarcoma in children. RMS is thought to derive from embryonic cells committed to developing into skeletal muscle and is divided into two major subtypes in childhood: embryonal and alveolar (eRMS and aRMS, respectively). The majority of aRMS (80–85%) contain one of the reciprocal chromosomal

Methods: The expression of HH ligands was studied by qPCR, western blot and immunohistochemistry. Functional and animal model studies were carried out with cells transduced with shRNAs against HH ligands or treated with HH-specific inhibitors (Vismodegib and MEDI-5304). Finally, the molecular characterisation of an off-target effect of Vismodegib was also made.

Results: The results showed a prominent expression of HH ligands supporting an autocrine ligand-dependent activation of the pathway. A comparison of pharmacologic Smoothened inhibition (Vismodegib) and HH ligand blocking (MEDI-5304) is also provided. Interestingly, a first description of pernicious off-target effect of Vismodegib is also reported.

Conclusions: The clarification of the HH pathway activation mechanism in RMS opens a door for targeted therapies against HH ligands as a possible alternative in the future development of better treatment protocols. Moreover, the description of a pernicious off-target effect of Vismodegib, via unfolded protein response activation, may mechanistically explain its previously reported inefficiency in several ligand-dependent cancers.
translocations: t(2;13)(q35;q14) or t(1;13)(p36;q14) (Barr et al., 1993; Davis et al., 1994). Conversely, no characteristic translocations have been described in eRMS, which is typically characterised by loss of heterozygosity on the short arm of chromosome 11 (11p15.5) (Loh et al., 1992) and gains in chromosomes 2, 7, 8, 11, 12, 13 and 17 (Bridge et al., 2000).

Hedgehog (HH) pathway-belonging proteins are considered to be key regulators of development. Likewise, HH signalling also plays important roles in adult organisms such as stem cell maintenance or tissue repair and regeneration. The three HH proteins present in mammals, Sonic (SHH), Indian (IHH) and Desert (DHH), are ligands of Patched receptors (PTCH1 and PTCH2). Ligand-free PTCH inhibits the activation of Smoothened (SMO) and Gli family zinc finger proteins (GLI1, GLI2 and GLI3) are proteosomically processed. Upon binding of an HH ligand, SMO becomes active and prevents GLI proteosomal processing. GLI is then translocated to the nucleus where regulates GLI-specific promoters to activate its specific targets (Hahn et al., 1998; Teglund and Toftgård, 2010).

HH signalling has been shown to be altered (either by mutation or deregulation) in many cancers including basal cell carcinoma (BCC), medulloblastoma, small-cell lung cancer and digestive tract tumours, among many others (Teglund and Toftgård, 2010). The relationship between HH signalling and RMS was first described in the Patched knockout mouse by Hahn et al. in 1998 who reported that mice heterozygous for Pch1 not only develop features consistent with Gorlin’s syndrome, but also have a high incidence of eRMS (Hahn et al., 1998). Consistent activation of the pathway is well established and generally accepted in RMS (Zibat et al., 2010; Pressey et al., 2011; Satheesha et al., 2015). However, controversy exists regarding the importance of mutation-driven constitutive activation of the pathway in RMS patients. Some works reported that neither PTCH mutations nor activating SMO mutations appear to be implicated in activation of the pathway (Calzada-Wack et al., 2002; Pressey et al., 2011); however, other authors reported losses in the PTCH region 9q22 in one third of eRMS and loss of SUPH has also been reported in 18% of eRMS (Bridge et al., 2000; 2002; Tostar et al., 2006). Additionally, genomic amplification of chromosomal region 12q13-15 containing GLI1 gene has been identified only in a subset of aRMS tumours (Bridge et al., 2002; Pressey et al., 2011). Consequently, the activator mutations reported to date can only account – in the best scenario – for the activation of the pathway in small subsets of patients. Moreover, a possible role of methylation of the PTCH1 promoter has been described and seems to be able to reduce WT allele expression in the Patched knockout mouse. However, this effect is only significant if concomitant with an oncogenic mutation of the first allele of this gene (Uhlmann et al., 2005), thereby reducing its potential in tumours with no PTCH1 mutation; therefore, the mechanism of activation remains elusive for the majority of patients.

On the other hand, several publications with xenografted RMS have raised the possibility of effectively reducing tumour growth (Eichenmüller et al., 2010; Tostar et al., 2010; Yamanaka et al., 2011) or even an impaired tumour initiation (Zibat et al., 2010) after pharmacologic inhibition of the pathway. In fact, small molecular antagonists of SMO entered clinical phase I and II trials for HH-driven neoplasias (Von Hoff et al., 2009; Lin and Matsui, 2012). Recently, in 2012, the SMO inhibitor Vismodegib was the first HH signalling pathway-targeting agent approved by the U.S Food and Drug Administration for the treatment of metastatic or locally advanced BCC (Abidi, 2014).

Despite the knowledge gathered to date, the mechanism that drives activation of the pathway in RMS is not well understood and the possible role of DHH or IHH in the pathogenesis of RMS has not been studied. Although activation of the pathway is well known in RMS, herein we report for the first time a preponderant expression of DHH and IHH ligands in RMS, which supports the existence of an autocrine ligand-dependent activation of the HH pathway in this neoplasia. The possible benefits of pharmacologic SMO inhibition (Vismodegib) and HH ligand blocking (MED15-5304) are also studied. Furthermore, the genetic silencing of the ligands led to a clear impairment of tumour growth of xenografted tumours. Finally, given that we observed a possible adverse effect of Vismodegib which promotes cell invasion in an HH-independent manner, the molecular characterisation of this putative undesired off-target effect is also provided.

**MATERIALS AND METHODS**

**Human samples.** Thirty-three frozen RMS tumours were obtained from our private collection (ISCIII C.0002311) and 20 from the CWS (‘Cooperative Weichteilsarkom Studiengruppe’) tissue bank (Stuttgart, Germany). Paraffin-embedded RMS tumour samples were obtained from our Pathology Department. Written informed consent according to the Declaration of Helsinki was obtained from all patients or their legal guardians and the study approved by the Vall d’Hebron Research Ethics Committee.

**Cell culture and drug treatment.** Cell lines RH30 and RH4 correspond to aRMS with PAX3/FOXO1 translocation, RH18 to a translocation-negative aRMS and RD and HTB82 to eRMS subtype. Some authors have identified the HTB82 cell line to a rhabdoid tumour since it bears a mutation in the SMARCBl gene (Khan et al., 2001; Hinson et al., 2013). All RMS cell lines were previously authenticated by STR-based DNA profiling, grown in MEM media (Biowest, Barcelona, Spain), supplemented with 10% foetal bovine serum (Sigma-Aldrich, Madrid, Spain), 2 mM L-glutamine, 1 mM sodium pyruvate and 1 x non-essential amino acids (all reagents from Biowest). All cell lines were obtained from American Type Culture Collection, except RH18 and RD cell lines which were a kind gift from Dr Beat Schäfer. The SMO inhibitor Vismodegib (GDC-0449) was purchased from Selleckchem (Madrid, Spain). The antibody MEDI-5304 was kindly provided by MedImmune (Cambridge, UK).

**RNA extraction, reverse transcription and real-time PCR.** Total RNA was extracted using the RNasy Mini Kit (Qiagen, Hilden, Germany). Two micrograms of total RNA were reverse-transcribed using 200 U of MMLV (Promega, Madrid, Spain) and random primers (Invitrogen, Thermo Fisher Scientific, Madrid, Spain). PCRs based on the TaqMan assay (Applied Biosystems, Thermo Fisher Scientific, Madrid, Spain) was performed to detect SHH, IHH, DHH and GLI1 (Hs00179843_m1, Hs00745531_s1, Hs00368306_m1 and Hs00171790_m1). The housekeeping gene TB (TATA-binding protein, Hs00172424_m1) was used as internal control. Relative levels of each mRNA were tested in triplicate and quantified by the method of Livak and Schmittgen (2001).

**Western blot.** Tumour samples were disrupted with a Pellet Pestle Motor (Sigma-Aldrich) in RIPA lysis buffer (Thermo Fisher Scientific, Madrid, Spain). Twenty micrograms of protein were separated on 8–15% SDS–PAGE gels and transferred to PVDF membranes (Life Sciences, Sigma-Aldrich, Madrid, Spain). Blotted membranes were blocked in 5% BSA and incubated with the appropriate antibodies: anti-SHH (1:2000, Millipore, Madrid, Spain), anti-IHH MABF23 (1:1000, Millipore), anti-DHH H00050846-B01P (1:500, Novus Biologicals, Abingdon, UK), anti-GLI1 clone EPR4523 (1:1000, Millipore), anti-GLI2 R770 (1:1000, Cell Signaling, Barcelona, Spain), anti-PTCH1 06-1102 (1:1000, Millipore), anti-Cyclin D2 sc-593 (1:200, Santa Cruz Biotechnology, Heidelberg, Germany), anti-TRIB3 antibody 07-2160 (1:1000, Millipore), anti-CHOP sc-7351 (1:200, Santa Cruz Biotechnology), anti-FAK #3285S (1:1000, Cell Signaling).
Plasmids and lentiviral transduction. Representative photographs of SHH, IHH and DHH immunohistochemistry (IHC) in archival paraffin-embedded human RMS tumour sections were shown for the alveolar cells (with the exception of RH30, with very low expression level). Concordantly with results in cell lines, SHH expression was negligible in the majority of RMS samples. However, approximately 30% of patients in a well-separated positive subpopulation showed remarkable mRNA SHH expression by real-time PCR (Figure 1C) and western blot (Figure 1D). In contrast, IHH and DHH levels were moderate, high or very high in all patients analysed by both qPCR (Figure 1C) and western blot (Figure 1D). No significant differences were observed between aRMS and eRMS (Supplementary Figure S1).

IHC in archival paraffin-embedded human RMS tumour sections also revealed predominant expression of DHH and IHH ligands in tumour cells. The majority of the tumours analysed showed very low or negligible SHH expression (15 out of 23, 65%), whereas the remaining 35% of samples showed low or moderate SHH levels (Figure 1H). Conversely, IHH and DHH were very highly expressed in the majority of samples, with a maximum expression of both proteins (semi-quantitative score 7) in 14 of 23 samples (61%), and semi-quantitative score values ≥ 4 for IHH in 18 of 23 samples (78%) and 20 of 23 samples for DHH (87%). Representative photographs of SHH, IHH and DHH immunohisto-chemistry (E, F and G, respectively) are shown in Figure 1 with two magnifications.

The presence of HH ligands correlates with activation of the pathway, thereby suggesting an autocrine mechanism. The presence of HH ligands in human RMS tumours was correlated...
Figure 1. IHH and DHH are widely distributed in RMS whereas SHH is only expressed in a small subset of patients. (A) mRNA levels of SHH, IHH and DHH in five cell lines (RH30, RH4, HTB82, RD and RH18). Values are expressed as $\Delta$CT value and the horizontal line depicts the mean of the values for the cell lines analysed. (B) Western blot showing the levels of the HH ligands in the same cell lines. The HEK cell line was used as a positive control. (C) Quantitative real-time PCR analysis of HH ligand expression in patients. Values are expressed as $\Delta$CT value and the horizontal line depicts the mean values ($n = 53$). (D) Western blot showing HH ligand levels in eight RMS patients (aRMS, alveolar RMS; eRMS, embryonal RMS). Tubulin was used as loading control (TUB). The presence of SHH (E), IHH (F) and DHH (G) in archival human samples was examined by immunohistochemistry at low magnification for a general view of the section (left), and the area indicated by a square is shown magnified on the right for a more detailed view. Bars: 100 μm. (H) Semi-quantitative evaluation of the expression of the three ligands in patient samples ($n = 27$). Horizontal lines depict the mean score for each marker.
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with the expression of one of the main downstream targets of the pathway, GLI1 (Figure 2A–D). The results showed significant correlation for SHH (P = 0.0197), DHH (P = 0.0268) and the sum of the three HH ligands (P = 0.0189). No significant correlation was obtained for IHH, although a trend was also observed. Furthermore, the genetic downregulation by shRNAs of the HH ligands and GLI1 (decrease of ligands and GLI1 is shown in Supplementary Figure S2) revealed a significant downregulation of some components and downstream targets of the pathway in the cell lines RD and RH4. Thus, IHH, DHH and GLI1 downregulation resulted in a marked decrease in GLI1, GLI2 and PTCH1, whereas SHH downregulation did not affect GLI1 expression but clearly decreased GLI2 and PTCH1 (Figure 2E and F). Conversely, in RH30 cell line that bears a GLI1 amplification none of the ligands significantly changed GLI1 expression. However, despite GLI1 amplification, the deprivation of SHH, IHH and DHH produced a significant reduction in GLI2 and PTCH1 expression (Figure 2G).

Deprivation of IHH and DHH rendered a reduction in cell proliferation. The effects of the shRNA-mediated IHH and DHH deprivation significantly reduced cellular proliferation of the cell lines RD and RH4 (Figure 3A and C). Unsurprisingly, no effects were observed for RH30 cells (Figure 3E). Interestingly, the pharmacologic effect of the HH ligand-blocking antibody MEDI-5304 produced comparable lessening in proliferation for RD and RH4. These cell lines were also sensitive to the SMO inhibitor Vismodegib and to the GLI1 shRNA. Once again, the RH30 cell line was completely insensitive to both ligand deprivation and MEDI-5304 antibody treatment, probably due to its GLI1 amplification. Only shRNA of GLI1 and Vismodegib treatment produced an antiproliferative effect on this cell line. Concordantly, Cyclin D2 levels were increased in cell lines RD and RH4 upon IHH, DHH and GLI1 deprivation (Figure 3B and D), thereby suggesting a cell cycle arrest at Gl1. No effects on this cell cycle effector were observed in the RH30 cell line (Figure 3F). Moreover, RD and RH4 RMS cells with downregulation of IHH and DHH showed a reduction in the percentage of cells in G2, thereby suggesting a cell cycle impairment (Figure 3G). Once again, the RH30 cell line showed no changes (not shown). Although a slight increase in early apoptotic cells and concordant PARP-1 cleavage was detected in the RD cell line, the results in the other two cell lines ruled out apoptosis as a general mechanism able to account for the decrease in the number of cells observed (Supplementary Figure S3). Moreover, no significant changes in total dead cells were observed for any of the cell line (not shown). The pharmacologic downregulation of the pathway was assessed by measuring Gli1 mRNA levels (Figure 3H–J).
Figure 3. Effects of the depletion of HH ligands on cell proliferation. (A, C and E) Relative proliferation, expressed as a reduction in WST uptake, compared to control cells (100%). The experiments were performed in the following samples: control (empty vector or vehicle) cells, cells transduced with shRNAs for SHH, IHH, DHH and GLI1 and, finally, cells treated with Vismodegib (50 μM) or the HH blocking antibody MEDI-5304 (30 μg/ml) after 4 days. The RMS cell lines analysed were RD, RH4 and RH30. (B, D and F) Western blots showing the accumulation of Cyclin D2 (CYC D2) in RD and RH4 cells. No changes were detected in RH30. (G) Cells transduced with shRNAs for IHH and DHH showed a slight reduction in the percentage of cells in G2, which indicates an impairment in the cell cycle. Percentages of cells in G1 and G2 + S are indicated in the plots. Significance: *P<0.05, **P<0.01, ***P<0.001. (H–J) Relative GLI1 mRNA expression after Vismodegib or MEDI-5304 treatment.
Genetic impairment of HH ligand expression produced a severe tumour growth reduction in SCID mice. Six and a half weeks after intramuscular RD cell injection, excised tumours stably expressing shRNAs against SHH, IHH, DHH and GLI1 were clearly smaller – or even in some cases were unable to grow – compared to the control tumours (empty vector) (Figure 4A). The average weight of the tumours was also clearly lower (Figure 4B). The difference was particularly dramatic after SHH and GLI1 downregulation (<25%) but also noteworthy for IHH and DHH downregulation (<40%). The kinetics of growth were clearly reduced in all cases (Figure 4C–F), with statistically significant differences from 5 to 6 weeks onwards. All shRNA targets were found to be downregulated as expected in tumours at the end point (Figure 4G).

**Figure 4.** Decreased tumour volume of RMS cells with genetically impaired expression of SHH, DHH, IHH and GLI1 in SCID mice. (A) Photograph of all the tumours showing the reduction in tumour size after genetic depletion of SHH, DHH, IHH and GLI1 (NTG: no tumour growth). (B) Average tumour weight at the end point of the study in comparison to the control (empty vector pGIPZ, 100% value). (C–F) Kinetics of tumour growth expressed as mm³ of tumour volume compared to empty vector for SHH, DHH, IHH and GLI1 shRNAs. Significance: *P<0.05, **P<0.01. (G) Western blot to assess the downregulation of HH ligands in tumours at the experiment end-point. shRNA-positive tumours (+) showed downregulation of their specific targets (SHH, IHH or DHH) compared with controls (–).

SHH played a crucial role for the invasive phenotype in ligand-dependent RMS cells and paradoxical enhancement of invasiveness by Vismodegib. In contrast to the effects seen on cell proliferation, the genetic inhibition of IHH and DHH produced no effects on cell invasiveness. Surprisingly, the effect of the downregulation of SHH in terms of invasiveness was noteworthy in RD and RH4 cell lines (Figure 5A and B). Once again the cell line RH30 was refractory to ligand deprivation (Figure 5C). The effects of MEDI-5304 antibody on invasiveness were statistically not significant. Unexpectedly, Vismodegib enhanced the invasive properties of all three cell lines by two-fold. These surprising results, together with the fact that the invasiveness of the RH30 cell line was likewise increased despite its GLI1-amplification (downstream of SMO), suggested an off-target effect of Vismodegib on cell invasiveness. Similar results were seen in mice treated with Vismodegib that were more prone to developing metastasis after a tail vein injection of RH30 cells (Figure 5D). Thus, in the control group (vehicle), only 55% of mice developed metastasis, whereas in the group treated with Vismodegib, all mice metastasised.

The paradox effect of Vismodegib on the invasiveness of RMS cells is mediated by off-target activation of the unfolded protein response pathway and concomitant phosphorylation of FAK. A cDNA microarray was performed to identify pathways differentially regulated between untreated and Vismodegib-treated cells. The expression profile analysis was made in three cell lines.
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Mutation-driven cancers such as those associated with Gorlin’s syndrome were the first in which an oncogenic role of the HH pathway was described (Hahn et al, 1996; Johnson et al, 1996). The archetypical molecular basis for the development of the malignancies associated with Gorlin’s syndrome is the PTCH1 loss of function which leads to constitutively activated HH signalling even in the absence of a ligand. Given that the mutation of components of the HH pathway in RMS appears to be rare (Calzada-Wack et al, 2002) – except in Gorlin’s syndrome that accounts for a very low percentage of RMS patients – a plausible hypothesis is that, regarding HH pathway activation, the majority of RMS are ligand-dependent tumours. To prove this hypothesis, it is crucial to demonstrate the presence of functional HH ligands in the tumours. However, an earlier report on HH ligand expression showed very low or undetectable SHH levels in RMS tumours and cell lines (Calzada-Wack et al, 2002), thereby pointing to a ligand-independent activation of HH signalling in RMS. We here show for the first time that IHH and DHH – and, in a subset of cases, also SIHH – are present in RMS tumour cells with concomitant variations in the expression of HH downstream targets, thus pointing to an autocrine mechanism of HH activation in which ligands may be both produced and responded to by the same tumour cells. Concordantly, the work of Satheesha et al (2015) ruled out paracrine activation of the pathway in RMS cells in in vivo mouse models. The reduction in proliferation upon IHH and DHH genetic deprivation in the cell lines RD and RH4 clearly indicates a crucial role of these ligands in the activation of the pathway and, therefore, is a further indication of ligand-dependent activation in these cell lines. The refractoriness of the cell line RH30, which bears a 10-fold GLI1 amplification (Calzada-Wack et al, 2002) is especially noteworthy. Presumably, GLI1 amplification makes the cell behave as ligand-independent, since the amplification bypasses the canonical ligand-dependent activation of the pathway. This cell line may be a paradigmatic example to illustrate the probable existence of a subset of patients with GLI1 amplification, as previously suggested (Calzada-Wack et al, 2002), which may be refractory to possible therapies based on ligand inhibition. According to that interesting previous work, GLI1 amplification seems to be alveolar-subtype specific (or at least is
Figure 6. Vismodegib treatment resulted in activation of the UPR pathway by FAK phosphorylation. (A) cDNA microarray analysis revealed overexpression of several components of the UPR pathway under Vismodegib (50 μM) treatment for 3 days compared to the control in three RMS cell lines (RD, RH4 and RH30). (B) Schematic view of the main components of the pathway. The elements found overexpressed in the microarray are shown in red. (C) Western blots showing the increase in CHOP and TRIB3 levels in RMS cell lines under Vismodegib treatment. (D and E) Western blots showing that Vismodegib treatment increased total and phosphorylated ATF4 as well as phosphorylation of FAK. (F) Assessment of TRIB3 downregulation by western blot after shRNA transduction in RD cell line. The selected shRNA (sh4) is indicated by an arrow. (G) The shRNA-mediated genetic inhibition of TRIB3 produced a very clear impairment in cell invasion (Matrigel/Transwell assay), whereas Vismodegib treatment alone produced a notable increase. The TRIB3 genetic downregulation alone produced only a slight reduction in cell invasion. Significance: *P<0.05, ** or #P<0.01, *** or ###P<0.001. (*) indicates differences to empty vector untreated control, whereas (#) indicates differences to treated empty vector.
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more frequently described in this subtype). However, the activation of the pathway in the majority of RMS tumours (including all eRMS and an undetermined proportion of aRMS) cannot be explained by this mechanism. On the other hand, cell lines RH4 and RD would represent tumours without mutations in HH pathway components, the oncogenicity of which strongly depends on ligand presence. The strong dependency on ligands, together with the fact that this phenotype presumably represents the majority of RMS patients, enables alternative therapeutic approaches based on ligand blocking in this neoplasia which may have wide translational potential.

The cell proliferation reduction drop observed in ligand-dependent cell lines after depletion of HH ligands strongly suggests that this is associated with a cell cycle alteration and the observed Cyclin D2 accumulation points to a G1-phase cell cycle arrest. Indeed, several of the genes activated by GLI proteins (i.e., Cyclin D) are key regulators of G1/S transition (Kasper et al., 2006). Interestingly, in the cell lines RD and RH4, the reduction in cell proliferation induced by pharmacologic inhibition is significant and comparable for both the ligand-blocking antibody MEDI-5304 and the SMO inhibitor Vismodegib. However, in RH30 cell line the inhibitory effect of Vismodegib on proliferation was probably an off-target effect, since GLI1 amplification is expected to result in insensitivity to SMO inhibitors. Conversely, the effect of MEDI-5304 appears to be pathway-specific since it did not impact on the RH30 cell line. Consequently, MEDI-5304 showed higher specificity and similar potency in the blocking of the HH pathway-induced proliferation. It is important to emphasise that the therapy of mutation-driven cancers by inhibition of the HH pathway constituents can only be successful if these are situated downstream of the acquired mutation or amplification (Heretsch et al., 2010). Conversely, ligand-dependent cancers are expected to be sensitive to therapeutic strategies based on ligand targeting. The results herein presented, as commented above, point to a reduction of RMS cell proliferation when treated with the antibody MEDI-5304. Interestingly, this antibody blocks SHH and IHH but fails to recognise DHH (Michaud et al., 2014). It is conceivable that the high level of expression of DHH in RMS may partially account for the moderate anti-proliferative effect of MEDI-5304. Thus, despite being the least active of the three HH ligands, DHH can significantly activate GLI1 expression (Pathi et al., 2001). Therefore, we cannot rule out that DHH in RMS plays a more important role than hitherto expected.

Although the shRNA-mediated downregulation of the ligands was not complete and performed separately for each ligand, the effects observed on tumour growth were remarkable. Thus, the downregulation of each single HH ligand clearly impaired tumour growth, suggesting that the participation of all ligands is crucial for RMS pathogenesis. The strong effect on tumour growth of SHH deprivation is particularly noteworthy. Because downregulation of SHH had no effect on cell proliferation in vitro, this ligand probably has to interact with the tumour microenvironment to execute its RMS-promoting functions. In this regard, the connection between an aberrant HH ligand secretion and the paracrine stimulation of stromal cells it is particularly interesting (Curran and Ng, 2008; Yauch et al., 2008). Indeed, also in RMS, SHH secretion may lead to a production of VEGF and IGF by the HH-stimulated stromal cells which, in turn, may affect angiogenesis of the growing tumour. Both factors, VEGF and IGF are clues to angiogenesis (Shigematsu et al., 1999; Bach, 2015).

The anti-oncogenic effect of the HH ligand deprivation is not restricted to cell proliferation. An essential role of HH pathway on epithelial to mesenchymal transition and/or invasiveness in a broad spectra of cancers (Lee et al., 2015; Lei et al., 2015; Liu et al, 2015) including RMS (Oue et al, 2013) has previously been well established. However, the possible influence of HH ligands on RMS cell invasion has not been elucidated to date. This work provides data to demonstrate for the first time a crucial role of SHH in the promotion of an invasive phenotype in RMS cells. Conversely, IHH and DHH appear not to influence invasion, pointing to a specific role of SHH in this aspect of tumour progression. Tumours with low expression of SHH had no effect on cell proliferation. In this regard, the connection between an aberrant HH ligand secretion and the paracrine stimulation of stromal cells it is particularly interesting (Curran and Ng, 2008; Yauch et al., 2008). Indeed, also in RMS, SHH secretion may lead to a production of VEGF and IGF by the HH-stimulated stromal cells which, in turn, may affect angiogenesis of the growing tumour. Both factors, VEGF and IGF are clues to angiogenesis (Shigematsu et al., 1999; Bach, 2015).

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Activation of the UPR pathway has been suggested to play a role in cancer (Ma and Hendershot, 2004). UPR is thought to be initiated by three endoplasmic reticulum (ER) stress sensors: protein kinase RNA-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1z (IRE1z) (Hetz et al., 2013). The fact that TRIB3, CHOP and ATF4 are essential downstream effectors of the pathway triggered by PERK and that they are activated (Figure 6) suggests that the activation of this response – after Vismodegib treatment – would be dependent on this UPR sensor, at least in RMS. To date, the potential UPR role in cancer is not completely understood and no relevant previous information on the implication of UPR in RMS progression was found; however, one of the most remarkable UPR functions in cancer progression is the promotion of invasiveness, including the promotion of some matrix metalloproteinas, extracellular matrix remodelling and angiogenesis (Dejeans et al, 2015). Interestingly, the concomitant increase in FAK phosphorylation (P-FAK) has been previously related to UPR activation as a mechanism able to induce cell survival, inhibit apoptosis and reduce chemosensitivity in cancer (Tang et al, 2012), thereby suggesting a pro-oncogenic potential associated with this mechanism. Moreover, the genetic inhibition of TRIB3 not only blocked the cell invasion increase but also promoted a clear sensitisation of cells to Vismodegib treatment, thereby pointing to a role for this protein in RMS chemoresistance. Taken together, the data reported and the discovery of this mechanism may help researchers and companies interested in the development and application of SMO inhibitors in cancer.

The clarification of the HH pathway activation mechanism in RMS, which has remained elusive to date, opens a door for targeted therapies against HH ligands as a possible alternative in the future development of better treatment protocols for RMS. Moreover, as a secondary point albeit not less important, the description of a pernicious off-target effect of Vismodegib may explain its previously reported inefficiency in several ligand-dependent cancers or even the premature halting of some of them owing to survival worsening. Therefore, the findings herein described may have significant translational implication not only for RMS but also for other HH ligand-dependent cancers.
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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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