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A role for the transcription factor HEY1 in glioblastoma

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Abstract

Glioblastoma multiforme (GBM), the highest-grade glioma, is the most frequent tumour of the brain with a very poor prognosis and limited therapeutic options. Although little is known about the molecular mechanisms that underlie glioblastoma formation, a number of signal transduction routes, such as the Notch and Ras signalling pathways, seem to play an important role in the formation of GBM. In the present study, we show by *in situ* hybridization on primary tumour material that the transcription factor HEY1, a target of the Notch signalling pathway, is specifically up-regulated in glioma and that expression of HEY1 in GBM correlates with tumour-grade and survival. In addition, we show by chromatin immunoprecipitations, luciferase assays and Northern blot experiments that HEY1 is a *bona fide* target of the E2F family of transcription factors, connecting the Ras and Notch signalling pathways. Finally, we show that ectopic expression of HEY1 induces cell proliferation in neural stem cells, while depletion of HEY1 by RNA interference reduces proliferation of glioblastoma cells in tissue culture. Together, these data imply a role for HEY1 in the progression of GBM, and therefore we propose that HEY1 may be a therapeutic target for glioblastoma patients. Moreover, HEY1 may represent a molecular marker to distinguish GBM patients with a longer survival prognosis from those at high risk.

Keywords: HEY1 • glioblastoma • E2F • Notch • RNA interference • molecular marker

Introduction

Gliomas represent the most common primary tumours of the brain and can be classified into different grades on the basis of their histology and degree of malignancy. The highest-grade glioma, named glioblastoma multiforme (GBM) carries a very poor prognosis and although much research has focused on the understanding of the development of GBMs, little is known about the cellular and molecular mechanisms that underlie glioblastoma formation. However, many cancer types, including gliomas, resemble undifferentiated cells in their gene expression and phenotypic characteristics [1] and it has been suggested that certain forms of GBM may arise by malignant transformation of glial precursor cells, such as the neural stem cells (NSCs) [2–4]. NSCs are pluripotent progenitor cells that have self-renewal activities and can generate various kinds of cell types within the central nervous system, including neurons, astrocytes and oligodendrocytes. The cell fate of NSCs is controlled by so-called positive regulators that induce cellular commitment and differentiation, and by negative regulators that keep the cell in an undifferentiated state. Negative regulators include growth factors such as fibroblast growth factor (FGF) and epidermal growth factor (EGF) [reviewed by 4, 5] whose

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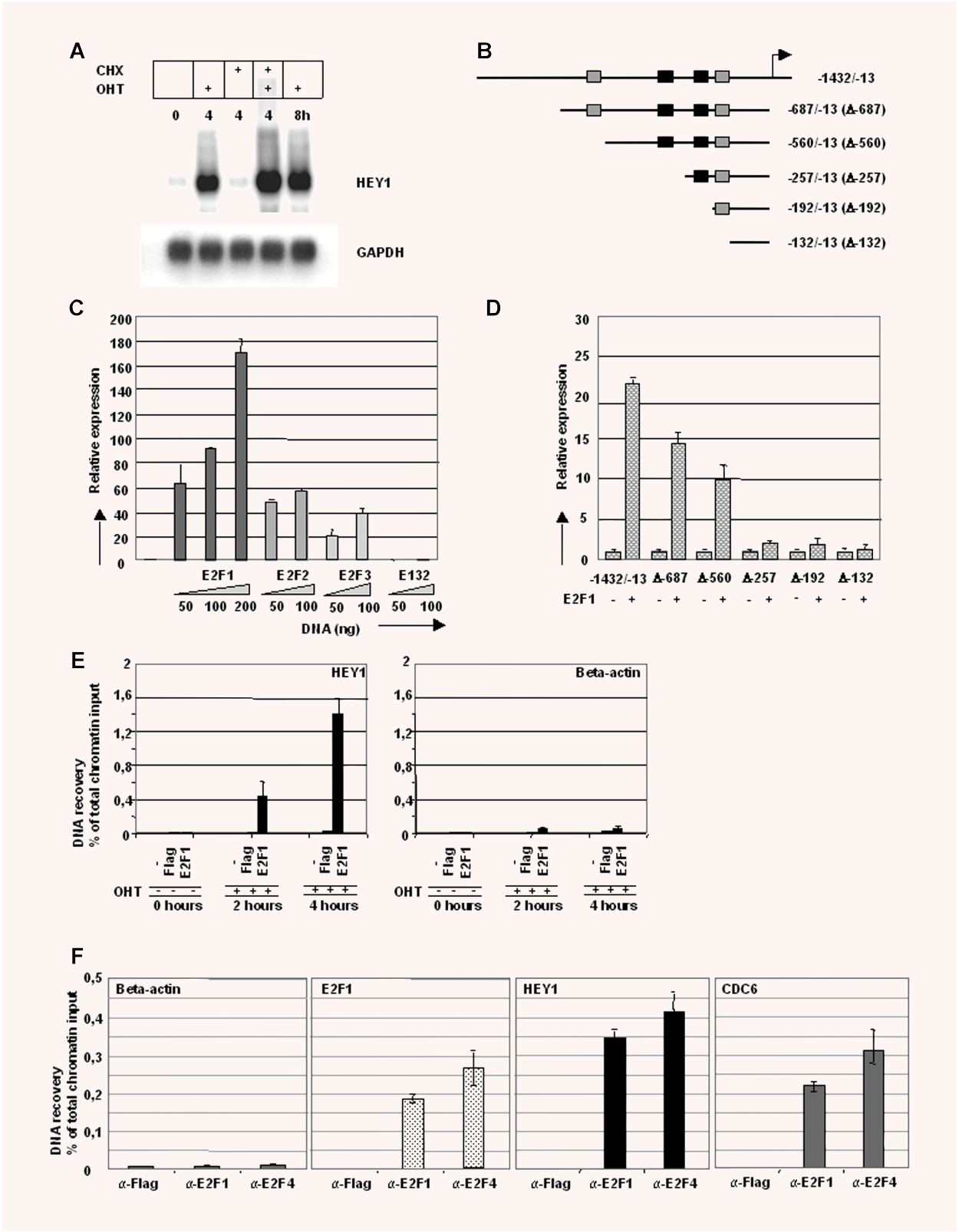

Fig. 1 HEY1 is a target of E2F transcription factors. (**A**) Northern blot showing induction of HEY1 expression upon activation of ER-E2F1 in WI38 cells. WI38 cells expressing the inducible ER-E2F1 construct were induced with 4-hydroxy tamoxifen (OHT) and/or cycloheximide (CHX) and harvested at the indicated times after treatment. Northern blot analysis was performed using 10 μg of total RNA. (**B**) Schematic representation of several truncation mutants of the *HEY1* promoter. Putative E2F-sites are indicated in black and additional RBP-Jk sites in grey. (**C**) Assay showing the luciferase activity driven by the full-length *HEY1* promoter upon transfection of increasing amounts of the constitutive active form of E2F1, E2F2 and E2F3 or the E2F1-DNA-binding mutant E132. The luciferase activity of the full-length HEY1 promoter without addition of E2F was taken to be 1 and relative activities were calculated. (**D**) Assay showing the luciferase activity of various 5′ truncations of the *HEY1* promoter, upon transfection of 100 ng of E2F1. The luciferase activity of each promoter in non-induced cells was taken to be 1 and relative activities were calculated. (**E**) Graphic representation of E2F1 present at the *HEY1*- or *β-actin* promoter after OHT treatment of TIG3 cells expressing the inducible ER-E2F construct as determined by chromatin immunoprecipitation. (**F**) Graphic representation of endogenous E2F1 and E2F4 present at the *HEY1*-, *E2F1*-, *CDC6*- or *β-actin* promoter in colo858 melanoma cells. Chromatin immunoprecipitation was performed by using the indicated antibodies. Immunoprecipitated promoter fragments were analysed by quantitative PCR to determine the relative presence of E2F1 and E2F4 on the indicated promoters as described in Materials and methods. A representative experiment is shown; data are presented as means ± SD ($n = 3$).

signalling pathways often are altered in high-grade gliomas [6–8], or components of the Notch signalling pathway.

The Notch signalling pathway is a conserved intercellular signalling route that has been implicated in different developmental processes. Members of the Notch gene family encode for transmembrane receptors that are activated through local cell–cell interactions: Notch receptors on the surface of one cell bind to their ligands on the surface of an adjacent cell, leading to the proteolytic cleavage of the Notch intracellular domain (NICD). The NICD subsequently translocates to the nucleus, where it can associate with the proteins of the RBP-Jκ family (also known as CSL or CBF/Su(H)/Lag-1) to form an active transcription factor complex that up-regulates the expression of primary target genes of Notch signalling, like the members of the hairy/Enhancer of split (E(spl)) family [reviewed by 9, 10].

Hairy/E(spl) family members, such as the HES or HEY proteins, are basic helix-loop-helix (bHLH) transcription factors that repress the expression of tissue specific genes and are involved in different developmental processes such as boundary formation, segmentation and the control of cell fate [11, 12]. One of the major processes in which several Hairy/E(spl) family members, are thought to be involved, is the process of neurogenesis [13–16]. Both HES and HEY family members inhibit the transcription of the proneural transcription factors Mash1, Math3, neurogenin and neuroD [14, 17] and recent findings suggest that Notch signalling plays an essential role in the formation of brain tumours and the self-renewal of NSCs [18–20]. However, the exact means by which Notch signalling contributes to brain tumour formation remains unclear, since activation of the Notch signalling pathway by overexpression of the NICD in immortalized astrocytes does not cause cellular transformation [20]. Moreover, the expression of HES and HEY proteins, both downstream of the Notch signalling route, can vary in different cell types, suggesting an additional mechanism of transcription regulation. One pathway that may contribute to this supplementary regulation is the pRB/E2F signal transduction route. The E2F transcription factors are involved in the control of various cellular processes, such as development, apoptosis and differentiation [reviewed by 21–23]. Several data indicate that pRB plays a crucial role in neurogenesis: pRB is highly expressed in the developing nervous system [24] and *Rb* deficient mouse embryos die between 13 and 15 days of gestation (E13–15) at the time when neural precursor cells normally initiate exit from the cell cycle and begin neuronal differentiation. In these mice, extensive apoptosis and differentiation defects in nervous tissue are observed and neuronal differentiation is impaired [25–28]. Moreover, neural precursor cells derived from $Rb^{-/-}$ embryos are found outside the normal neurogenic region, exhibit a delay in cell cycle withdrawal, an increase in S-phase population and deregulated E2F activity [29, 30]. In addition, increased cell division has been described in telencephalon-specific *Rb* knockout mice and in conditional *Rb* mutants, leading to an increase in brain size [31, 32]. The importance of the pRB/E2F signalling pathway in neural and glial differentiation is further underscored by the observation that alterations in the pRB/E2F pathway are found in several types of brain tumours, including gliomas of different grades [2, 33–35].

Here, we show that the E2F transcription factors directly regulate the expression of HEY1 and that overexpression of HEY1 in NSCs induces proliferation, while impairment of HEY1 expression in glioblastoma cells in tissue culture results in a reduction of proliferation. Furthermore, we demonstrate that HEY1 is specifically overexpressed in glioma, and that expression correlates with survival and tumour grade. These data suggest that HEY1 may play a role in the development of brain tumours and, as such, HEY1 might represent a molecular marker or a therapeutic target for the treatment of GBM.

Materials and methods

Cell culture and retroviral infections

Human WI38, U2OS, colo858, TIG3, phoenix cells and U-87 MG, U-373 MG, T98G glioma cells were cultured at 37°C in a 5% humidified atmosphere in Dulbecco's Modified Eagle Medium (DMEM) or Roswell Park Memorial Institute (RPMI) (colo858) plus 10% foetal calf serum. NSCs were isolated from 2-day-old wild-type C57Bl6 mice and cultured as described previously [36]. Pools of early passage WI38 or TIG3 ER-E2F1 cells were generated by infection with the retroviral vector pBabePuro ER-E2F1 as described earlier [37] and selected in 1.5 μg/ml puromycin. E1A- or HEY1-expressing NSCs

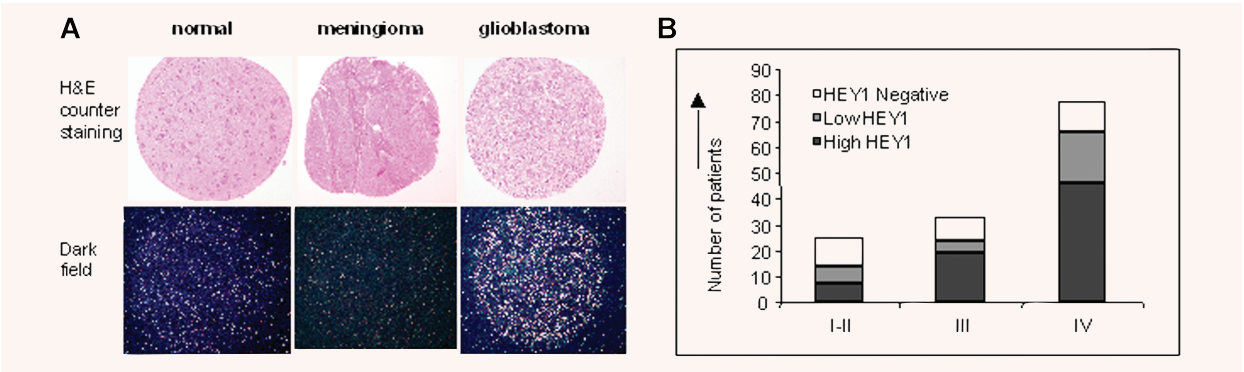

Fig. 2 HEY1 is specifically expressed in glioblastoma. (**A**) Representative images of HEY1 expression as detected by *in situ* hybridization-tissue microarray (ISH-TMA); in each pair, the bright field haematoxylin and eosin counterstaining for morphologic evaluation (upper panel, 100× magnification) and the concomitant dark field HEY1 ISH signal (silver grains, lower panel) are shown. Transcripts appear as bright dots; the signal observed in normal brain is considered to be background staining. (**B**) Summary of HEY1 expression on glioma specific TMAs as determined by *in situ* hybridization; the number of HEY1 positive tumours is shown in relationship to tumour grade.

Table 1 Summary table of HEY1 expression in different tumour types as determined by *in situ* hybridization on three multi-tumour TMAs

Organ	Type	Incidence	Proportion
Breast	Carcinoma	0/23	
Colon	Carcinoma	0/25	
Lung	Carcinoma	0/20	
Prostate	Carcinoma	0/15	
Stomach	Carcinoma	0/14	
Uterus	Carcinoma	0/28	
CNS	Glioblastoma	5/13	38%
Skin	Melanoma	3/15	20%
Soft tissue	Sarcoma	0/16	
Haemat. tissue	Lymphoma	0/15	

were generated by infection with the pBabe retroviral vector, containing the coding sequence of E1A or human *HEY1* and selected in 1 μg/ml puromycin.

Northern blot analysis

Nearly confluent cultures of WI38 cells expressing ER-E2F1 were trypsinized and plated at 5×10^{-6} cells per 15 cm plate on the day before induction. The ER-E2F1 fusion protein was activated by addition of 4-hydroxytamoxifen (OHT) to a final concentration of 300 nM and samples were harvested at the indicated times after induction. Cycloheximide was added where indicated to a final concentration of 10 μg/ml. RNA was isolated using the Qiagen RNeasy kit and 10 μg of total RNA was separated on 1.25% formaldehyde agarose gels, transferred to a Hybond N^+ membrane (Amersham, Buckinghamshire, UK). Probe used for the Northern blot was spanning the *HEY1* coding region from nucleotide +190 to +418.

Cloning of HEY1 and the HEY1 promoter and luciferase assays

The *HEY1* DNA sequence was retrieved from the NCBI database and primers were designed to amplify the entire gene or the 5′ upstream region, from cDNA and genomic DNA respectively. The PCR products obtained were subsequently cloned in the TA-TOPO vector (Invitrogen, Carlsbad, CA, USA) and verified by sequencing. Applying the same strategy, different mutants of the *HEY1* promoter were obtained and, like the full-length *HEY1* promoter, cloned into the pGL3 basic luciferase vector (Promega, Madison, WI, USA). U2OS cells were subsequently transfected with 200 ng of luciferase reporter constructs (pGL3-HEY1, full-length or mutants), 200 ng of pCMVβ-Gal reporter construct and different amounts of plasmid DNA to adjust the total amount of DNA to 2 μg. Different E2F constructs were co-transfected at the concentrations indicated in the figure legends. Two days after transfection, cells were harvested for luciferase and β-galactosidase activity and normalized as described previously [38].

Chromatin immunoprecipitation assays

Chromatin immunoprecipitations were performed as described previously [39]. Briefly, colo858 or TIG3 ER-E2F1-expressing cells were cross-linked by addition of 1% formaldehyde and treated with 2 M glycine. Cells were washed twice in tris buffered saline (TBS) (20 mM Tris/HCl [pH 7.4], 150 mM NaCl) and harvested in SDS buffer. After centrifugation, cells were re-suspended in immunoprecipitation buffer and sonicated. Lysates were subsequently pre-cleared with protein A sepharose beads (Amersham) and incubated with antibodies specific for E2F1 (SC-193), E2F4 (SC-866) or with an unrelated Flag antibody (F3165 Sigma).

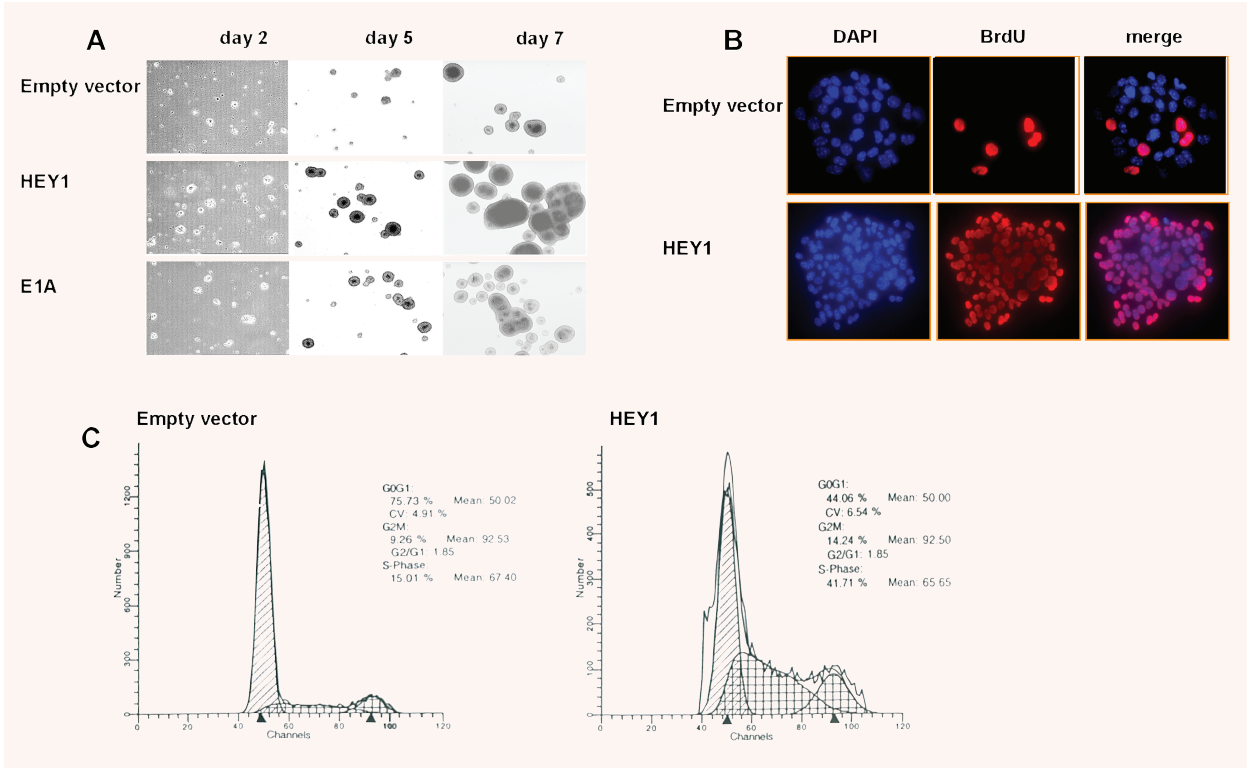

Fig. 3 Changes in HEY1 expression alter the rate of cell proliferation. (**A**) Neural stem cells (NSCs) infected with a retroviral vector expressing HEY1 form bigger neurospheres than NSCs infected with empty vector or E1A. (**B**) Immunofluorescence showing an induction of BrdU incorporation in non-differentiated NSCs. Non-infected (upper panels) or HEY1 infected (lower panels) NSCs were incubated for 24 hrs in the presence of BrdU and fixed on glass cover slips by cytospin-treatment. Cells were subsequently stained with DAPI or an antibody specific for BrdU. (**C**) FACS profiles of NSCs infected with a retroviral vector expressing HEY1 as compared to NSCs infected with empty vector.

Table 2 Summary table of HEY1 expression on CNS and glioma specific TMAs as determined by *in situ* hybridization

Type	Incidence	Proportion
Normal (Haemorrhage)	0/10	-
Meningioma	0/32	-
Glioma	104/136	76.5%
Neuroblastoma	0/68	-
Ependymoma	1/14	-
Other brain tumours	0/19	-
Metastases	1/8	-

Immunocomplexes were recovered with protein A sepharose beads and extensively washed. Cross-links were subsequently reversed after proteinase K treatment and DNA was recovered by a phenol chloroform extraction followed by ethanol precipitation. DNA was re-suspended in 150 µl water and 7.5 µl was subsequently used for a 25 ml Q-PCR reaction. Primer sequences are available upon request.

Tissue microarray (TMA) construction and *in situ* hybridization (ISH)

For the large-scale screening study, formalin fixed and paraffin-embedded tumour and normal specimens were provided by the Pathology Departments of the Ospedale Maggiore (Novara, Italy), Presidio Ospedaliero (Vimercate, Italy) and Ospedale Sacco (Milano, Italy). Samples were arrayed in three different multi-tumour TMAs as previously described [40]. Briefly, for each sample, two 0.6 mm cylinders from both tumour and normal counterpart tissue (when available) were taken and deposited on the recipient block using a custom-built precision instrument (Tissue Arrayer—Beecher Instruments, Sun Prairie, WI 53590, USA). Two micrometer sections of the resulting recipient block were cut, mounted on glass slides and processed forISH. For the in-depth brain tumour analysis, specimens from the Istituto Clinico Humanitas (Milan, Italy), Ospedale san Paolo (Milan) and Erasmus

Medical Center (Rotterdam, The Netherlands) were spotted in 2 additional specific TMAs.

HEY1 mRNA expression was assessed by ISH using [35S] UTP-labelled sense and antisense riboprobes [41]. TMA sections were deparaffinized, digested with proteinase K (20 μg/ml), post-fixed, acetylated and dried. After overnight hybridization at 50°C, sections were washed in 50% formamide, 2X saline sodium citrate (SSC), 20 mM 2-mercaptoethanol at 60°C coated with Kodak nitrobluetetrazolium (NBT)-2 photographic emulsion and exposed for 3 weeks. The slides were lightly haematoxylin and eosin counterstained and analysed at the microscope with a dark field condenser for the silver grains. All TMAs were first analysed for the expression of the housekeeping gene β-*actin* to check for the mRNA quality of samples. Cases showing absent or low β-actin signal were excluded from the analysis. Gene expression levels were evaluated by counting the number of grains per cell and were expressed in a semi-quantitative scale (ISH score): 0 (no staining), 1 (1–25 grains: weak staining), 2 (26–50 grains: moderate staining) and 3 (>50 grains: strong staining). ISH scores 2 and 3 were considered to represent an unequivocal positive signal.

Immunofluorescence and FACS analysis

For bromodeoxyuridine (BrdU) incorporation studies, NSCs were pulsed with 33 μm BrdU for 24 hrs and cytospins containing neural spheres were prepared by centrifuging 50 μl of the NSC suspension at 300× *g* for 7 min. (Heidolph Shandon cytospin, Shandon Scientific Ltd, UK). Cells were washed in PBS, fixed in 4% formaldehyde for 1 hr and incubated with anti-BrdU antibody (BectonDickinson) and 4′,6-diamidino-2-phenylindole (DAPI). Between each incubation step, coverslips were washed with PBS. For flow cytometric analysis, cells were washed in PBS, dissociated to single cells and fixed by addition of ice-cold ethanol to a final concentration of 75%. The cells were washed once in phosphate buffered saline (PBS) and re-suspended in PBS containing 50 μg/ml of propidium iodide, 250 μg/ml of RNaseA and incubated overnight at 4°C. The cell cycle profiles were subsequently analysed with a Becton Dickinson FACScan flow cytometer.

RNA interference and quantitative real time PCR

Specific siRNA oligos targeting *HEY1* mRNA and a non-specific oligo targeting the luciferase gene were designed as indicated by Dharmacon Research. Logarithmically growing U-87 MG, U-373 MG and T98G glioma cells were seeded at a density of 8×10^4 cells/well in 6-well plates in serum-containing medium. Cells were transfected 24 hrs later in serum-free medium using Oligofectamine (Invitrogen) according to the supplier's protocol. At the indicated time-points, cells were counted in triplicate and lysed for RNA isolation with the RNeasy protocol (Qiagen, Venlo, The Netherlands). cDNA was synthesized using superscript II, 5 μg of total mRNA template and random hexamers as primers, as described by the manufacturer (Gibco BRL/Life Technologies, Grand Island, NY, USA). Quantitative RT-PCR analysis was performed on the Perkin-Elmer/Applied Biosystems Prism 7700 Sequence Detection System (Foster City, CA, USA) by monitoring the increase of fluorescence by binding of SYBR green to double-stranded DNA. PCR primers were designed with Primer Express software (Applied Biosystems) and spanned exon junctions to prevent the amplification of any possible contaminating genomic DNA. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control gene for normalization. Primer sequences are available upon request.

Results

HEY1 is a target of the E2F family of transcription factors

Although initially identified as a direct target of the Notch signalling pathway [42, 43], the Hairy/E(spl) transcriptional repressor HEY1 was identified as a putative E2F-regulated gene in a high-density oligonucleotide screen performed previously in our laboratory [38]. To establish if *HEY1* is a physiological target gene of the E2Fs, several experiments were carried out. First, Northern blot analysis was performed using total RNA prepared from a human diploid fibroblast cell line, WI38, which expresses E2F1 fused to the ligand-binding domain of the oestrogen receptor [37, 38, 44]. This fusion protein is expressed as an inactive form in the cytoplasm and is activated by OHT, resulting in a translocation to the nucleus. As shown in Fig. 1A, *HEY1* mRNA levels are highly increased upon E2F1 activation in WI38 cells. This up-regulation could be seen also in the absence of *de novo* protein synthesis, as confirmed by addition of cycloheximide during OHT treatment, suggesting that *HEY1* is a direct transcriptional target of E2F1. Next, we cloned the promoter of the human *HEY1* gene. Besides the already published RBP-Jk sites that are responsible for response to Notch signalling [45], two E2F DNA-binding sites were identified at position −357 and −234 respective to the start site of transcription (Fig. 1B and supplementary Figure 1). In order to test the functionality of the putative E2F-binding sites, the *HEY1* promoter and several truncated mutants were used in luciferase reporter assays. Ectopic expression of E2F1, E2F2 and E2F3, but not the E2F1 (E132) DNA-binding mutant, induced transcription driven by the longest version of the HEY1 promoter (Fig. 1C) in human U2OS cells, whereas removal of the potential E2F DNA-binding site at −357 respective to the start site of transcription rendered the promoter unresponsive to ectopic expression of E2F1 (Fig. 1D).

Once identified the E2F-binding sites in the promoter of *HEY1* and having demonstrated that the activation of *HEY1* transcription depended on the presence of these binding sites, we wanted to show direct binding of E2F to the promoter. For this purpose two different types of chromatin immunoprecipitation (ChIP) assays were performed. In the first experiment, DNA from TIG3 cells expressing the inducible ER-E2F1 protein was precipitated using an antibody specific for E2F1. As shown in Fig. 1E, E2F1 associates with the *HEY1* promoter, but not the β-actin promoter, after activation of ER-E2F1. The specificity of this interaction was further controlled by using a non-related (Flag) antibody. Next, we examined the binding of endogenous E2F transcription factors to the *HEY1* promoter. Therefore, chromatin of the melanoma cell line colo858 was precipitated using antibodies specific for E2F1 and E2F4. Both E2F antibodies precipitated promoter fragments from the known E2F target genes, *E2F1* and *CDC6*. Moreover, both E2F1 and E2F4 also precipitated the previously identified E2F-binding sites in the *HEY1* promoter in these cells, confirming that physiological levels of the E2F transcription factors are associated with the *HEY1* promoter. Again, no chromatin

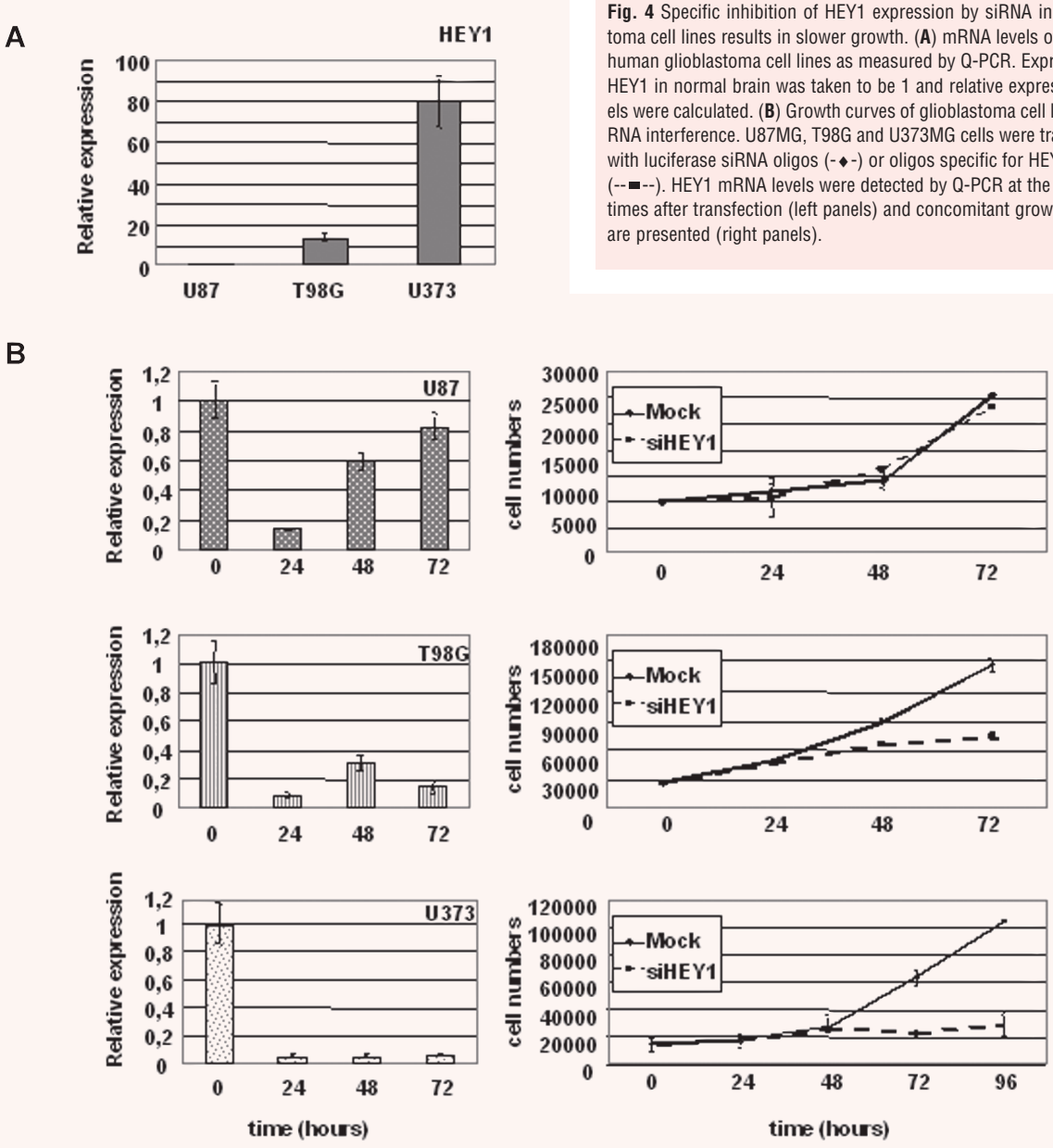

Fig. 4 Specific inhibition of HEY1 expression by siRNA in glioblastoma cell lines results in slower growth. (**A**) mRNA levels of HEY1 in human glioblastoma cell lines as measured by Q-PCR. Expression of HEY1 in normal brain was taken to be 1 and relative expression levels were calculated. (**B**) Growth curves of glioblastoma cell lines after RNA interference. U87MG, T98G and U373MG cells were transfected with luciferase siRNA oligos (-♦-) or oligos specific for HEY1 mRNA (--■--). HEY1 mRNA levels were detected by Q-PCR at the indicated times after transfection (left panels) and concomitant growth curves are presented (right panels).

Table 3 Summary table of the disease-free survival (DFS) and median survival of glioblastoma patients expressing or non-expressing HEY1. In all experiments, investigators blinded to the clinical outcome performed *in situ* hybridizations

	HEY negative	C.I.95%	HEY positive	C.I.95%
Overall survival (months)	21.67	9.04–34.29	10.87	8.64–13.10
Disease-free interval (months)	18.25	7.09–29.4	8.97	6.76–11.17

precipitation was observed when a non-related antibody was used and no E2F binding to the β-actin gene was detected (Fig. 1F). Taken together, our results show that HEY1 is a bona fide E2F target gene.

Expression of HEY1 in primary human tumours

Since the pRB/E2F signal transduction pathway is frequently deregulated in different kinds of tumours and accumulating results indicate that deregulated Notch activity is also involved in the genesis of human cancers [46, 47], we set out to determine the expression of HEY1 in primary human tumours. Therefore, primary human tumour material, representing 10 different types of tumours was spotted on tissue micro arrays (TMAs) – together with their normal counterparts – and screened by ISH to determine *HEY1* mRNA expression. As shown in Table 1, expression of HEY1 was not detected in most of the normal and tumour tissues analysed, but HEY1 was highly expressed in several melanomas and glioblastomas. Of the 13 glioblastoma samples present on the TMA, one tumour showed no detectable HEY1 mRNA expression, seven samples expressed low and five samples very high amounts of HEY1 mRNA. Similarly, HEY1 was found to be highly expressed in about 20% of melanoma samples tested (3/15). The high expression of HEY1 in glioblastoma was particularly interesting, since several Hairy/E(spl) family members have been shown to be involved in neurogenesis [48–50]. Moreover, recent findings suggest that Notch signalling plays an essential role in the formation of brain tumours and the self-renewal of NSCs [51–54]. Therefore, 170 additional brain tumour samples – including the fullrange of malignancy grades from low-grade gliomas to GBM – were examined for HEY1 expression using ISH. Strikingly, HEY1 expression was detected almost exclusively in glioma, while no expression was found in other brain tumours or in normal brain tissue (Fig. 2A, Table 2). Since, in these cases, no normal counterparts of the same patients were available, other brain biopsies were used as a negative control (haemorrhage).

Interestingly, when evaluating the levels of HEY1 expression by the *in situ* analysis, HEY1 expression was found to correlate with tumour grade. The number of tumour samples that expressed HEY1, the number of cells within a tumour sample that expressed HEY1 and the intensity of the staining increased from lower grade astrocytic glioma and oligodendroglioma to high-grade GBM. HEY1 expression was detected in about 45% of grade II or III astrocytoma and oligodendroglioma, while moderate-to-high expression of HEY1 was detected in almost 60% of glioblastoma (grade IV), adding up to 85% when also low-expressing tumours were included (Fig. 2B). Statistical analysis using the Kruskal–Wallis test showed a significant correlation between tumour grade and expression of HEY1 ($P = 0.0088$). More importantly, however, expression of HEY1 was also found to correlate with clinical outcome and survival. In general, patients with GBM have very bad prognosis with a mean survival of about 1 year. Univariate analysis of the clinical outcome of 62 cases of GBM present on the TMA demonstrated that patients expressing HEY1 survived nearly 1 year, while HEY1 negative patients survived two times longer ($P = 0.0037$) (Table 3). Correspondingly, HEY1-expressing patients had a significantly shorter median disease-free survival (DFS) when compared to HEY1 negative patients (8.9 *versus* 18.3 months respectively [$P = 0.0053$]). This finding and the observation that no expression was detected in normal brain tissue, indicates that HEY1 is a significantly unfavourable prognostic factor in the survival of patients with glioblastoma.

Role of HEY1 in glioma cell proliferation

To determine whether HEY1 might contribute to the development of GBM, full-length HEY1 was ectopically expressed in NSCs isolated from wild-type C57/Bl6 mice. As shown in Fig. 3A, overexpression of HEY1 led to the formation of very large neurospheres when compared to control cells. Overexpression of the adenovirus E1A protein also led to the formation of large colonies and was used as a positive control. Since the big neurospheres could have arisen by increased cell proliferation or by aggregation of smaller spheres, we determined whether HEY1 expression leads to an increase in DNA replication. NSCs were labelled with BrdU for 24 hrs and, as shown in Fig. 3B, NSCs infected with a plasmid expressing HEY1 show a clear increase in BrdU positive cells as compared to cells infected with an empty vector. The number of cells in G0/G1 phase decreased from about 76% in the control to 44% of HEY1-infected cells and, consistently with this, more HEY1-expressing cells were found in the S phase of the cell cycle when cells were infected with an HEY1-expressing vector (15% *versus* 42% respectively, Fig. 3C).

To test if HEY1 expression is required for the maintenance of glioblastoma cell proliferation and as such may represent a candidate drug target, we transfected various glioblastoma cell lines, expressing different levels of HEY1 (Fig. 4A), with a siRNA specific for HEY1. Although the endogenous levels of HEY1 mRNA were relatively high in some of the cell lines used, HEY1 expression was not detectable by Western blotting using the currently available antibodies. Thus, in order to check the efficiency of the RNA interference, HEY1 expression was examined by real time Q-PCR. As shown in Fig. 4B, HEY1 expression in the glioblastoma cell line U87MG was drastically decreased after 24 hrs of treatment with siRNA oligos, but levels increased rapidly at later time-points. Inhibition of HEY1 expression in the cell lines T98G and U373, which have relatively high levels of HEY1, was more effective and persisted for at least 72 hrs. The decrease in HEY1 expression correlated with a reduction in cell proliferation, demonstrating that HEY1 is required for the proliferation of glioblastoma cells with high expression of HEY1.

Discussion

Here, we have shown that HEY1 is a novel target of the E2F transcription factors and that HEY1 has a potential role in the progression of brain tumours. High levels of HEY1 mRNA were detected in human glioblastoma using ISH on TMAs – performed

in a blinded fashion – and were confirmed by Q-PCR analysis on an independent set of GBM samples (data not shown).

At present, we do not know the exact molecular events leading to high levels of HEY1 expression in GBM, but it is tempting to speculate that the expression of HEY1 observed in glioma might be a consequence of increased E2F activity. FISH analysis, using a probe specific for the *HEY1* locus (8q21) did not reveal any chromosomal abnormalities (data not shown) even though this area of the genome often is found amplified in different types of tumours, including gliomas [55]. Moreover, different members of the E2F signalling pathway have previously been found to be altered in human glioblastoma [reviewed by 2] and down-regulation of E2F1 activity has been shown to induce cell cycle arrest in glioblastoma cell lines [35]. However, HEY1 has previously also been identified as a downstream target of the Notch signal transduction route [12, 42–43] and several groups have recently reported the importance of Notch signalling in gliomagenesis [20, 53, 56]. Thus, it may well be that the high levels of HEY1 expression are a consequence of both altered E2F and Notch signalling. In fact, many gliomas exhibit deregulated Ras and Notch activity and it has been suggested that both pathways may be required for GBM formation [20, 53] and that Ras and Notch may co-operate directly to specify a particular cell fate by up-regulating a common target gene [57].

Although HEY1 may not necessarily be the 'missing link' between Ras and Notch signalling, and both pathways may have several downstream targets that are involved in gliomagenesis, the results presented here suggest that HEY1 is an important factor for the formation of GBM. Overexpression of HEY1 in NSCs isolated from newborn mice increased DNA synthesis and cell proliferation resulting in the formation of large neurospheres – an effect that was also observed upon overexpression of E1A, which is known to possess potent transformation activity. The exact mechanism by which HEY1 induces cell proliferation is not clear; HEY1 has been reported to promote the maintenance of NSCs and to repress the expression of pro-neuronal transcription factors such as Mash1, neurogenin and neuroD [14], but since the NSCs were grown under conditions that repress differentiation, the effects of HEY1 on proliferation do not seem to be due to inhibition of differentiation-specific transcription factors. In fact, when NSCs are induced to differentiate by removal of growth factors [36], HEY1-expressing cells differentiate into astrocytes, oligodendrocytes and neurons (results not shown). Interestingly, high levels of HEY1 were also observed in stem cell-like precursors isolated from a glioblastoma patient (data not shown). Moreover, the inhibition of HEY1 expression by the use of siRNA significantly decreased the proliferation of glioblastoma cell lines with high levels of HEY1. Although the use of glioma cell lines does not necessarily represent the situation in fresh tumour cultures, these results clearly show that HEY1 contributes to the proliferation of established glioblastoma cell lines.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

A role for the transcription factor HEY1 in brain tumour development Esther Hulleman, Micaela Quarto, Richard Vernell, Giacomo Masserdotti, Elena Colli, Johan M. Kros, Daniel Levi, Paolo Gaetani, Patrizia Tunici, Gaetano Finocchiaro, Riccardo Rodriguez y Baena, Maria Capra[2] and Kristian Helin

Fig. S1 The HEY1 promoter contains putative E2F binding sites. Sequence of the full-length human HEY1 promoter containing 2 putative E2F binding sites at position –234 and –357 respective to the start site of transcription. RBP-Jk sites indicated in white boxes, E2F binding sites indicated in gray boxes.

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