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Direct interaction of Gas41 and Myc encoded by amplified genes in nervous system tumours

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In order to understand better the role of the human Tip60 complex component Gas41, we analysed its expression levels in brain tumours and searched for possible interactors. Two-hybrid screening of a human foetal brain library allowed identification of some molecular interactors of Gas41. Among them we found n-Myc transcription factor. The interaction between Gas41 and n-Myc was validated by pull-down experiments. We showed that Gas41 is able to bind both n-Myc and c-Myc proteins, and that the levels of expression of Gas41 and Myc proteins were similar to each other in such brain tumors as neuroblastomas and glioblastomas. Finally, in order to identify which region of Gas41 is involved in the interaction with Myc proteins, we analysed the ability of Gas41 to substitute for its orthologue Yaf9 in yeast; we showed that the N-terminal portions of the two proteins, containing the YEATS domains, are interchangeable, while the C-terminal portions are species-specific. In fact we found that Gas41 C-terminal portion is required for Myc protein interaction in human.

Keywords: Gas41, n-Myc, transcription regulation, chromatin modification, brain tumours

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INTRODUCTION

The glioma-amplified sequence (*gas*) *41* gene was identified for the first time as an amplified sequence in the human chromosome region 12q13-15, a locus known to be involved in gene amplification in human gliomas. Genomic amplifications are frequently found in human tumours and thought to be an indication of the intrinsic genome instability of the cancer cells. This gene was found to be amplified in 23% of glioblastomas and in 80% of grade I astrocytomas, suggesting that gene amplification occurs also in early stages of cancerogenesis (Fischer *et al.,* 1996; 1997; Zimmermann *et al.,* 2002). Gas41 is a highly conserved protein (Harborth *et al.,* 2000; Zimmermann *et al.,* 2002; Le Masson *et al.,* 2003). Sequence comparison indicates high homology of Gas41 with the human transcription factors AF-9 and ENL. Differently from ENL and AF-9, Gas41 does not have a typical DNA binding domain, suggesting a mediated interaction with DNA targets. Gas41 has a nuclear localization and interacts with many complexes and proteins: the nuclear mitotic apparatus (NuMa), the prefoldin-like protein subunit 1 (PFDN1), the KIAA1009 protein and the MLL-AF10 fusion protein, which is detected in 5–10% of human acute leukaemias (Debernardi *et al.,* 2002).

Particularly informative for the function of *gas41* are studies on its yeast homologous gene *YAF9* (Yeast AF-9). *YAF9* encodes a 226-aminoacid protein with 53% identity and 80% similarity with Gas41. Most of the homology is in the N-terminal part (Fischer *et al.,* 1997; Le Masson *et al.,* 2003). *YAF9* is a non-essential yeast gene but the *yaf9*Δ strain shows various growth phenotypes (Bianchi *et al.,* 2001). Yaf9 is involved in transcriptional regulation of several genes (Le Masson *et al.,* 2003; Bianchi *et al.,* 2004; Del Vescovo *et al.,* 2008; Casagrande *et al.,* 2009) and binds to regulated promoters, affecting histone acetylation levels (Bianchi *et al.,* 2004). Yaf9 is present in the NuA4 histone acetyltransferase complex (Bianchi *et al.,* 2004) and in the SWR1-complex (SWR1-C) involved in the substitution of histone H2A with H2AZ (Krogan *et al.,* 2003; Mizuguchi *et al.,* 2004; Zhang *et al.,* 2005).

In human, Gas41 is a common subunit of the SRCAP and Tip60 complexes which are the mammalian version of SWR1 and NuA4 complexes, respectively; also in *Drosophila*, Gas41 is involved in a complex which appears to correspond to NuA4 and SWR1 yeast complexes (Doyon *et al.,* 2004; Doyon & Cote 2004; Schultze *et al.,* 2009). These complexes change chromatin structure and allow gene transcription, suggesting that Yaf9 and Gas41 might promote the recruitment of these complexes to regulated promoters in yeast and mammalian cells, respectively. Interestingly, Yaf9 seems be required also for the cellular response to spindle stress in yeast, a function correlated to the recruitment of NuA4 to the centromeric chromatin (Le Masson *et al.,* 2003; Krogan *et al.,* 2003). On the other hand, Gas41 has been shown to interact with INI (Integrase Interactor 1) which is the human homologue of the yeast SNF5, a component of the SWI/SNF chromatin remodelling complex (Debernardi *et al.,* 2002), and with TACC1 and TACC2 proteins (Gangisetty *et al.,* 2004). TACC proteins are involved in the control of cell growth and differentiation and might be implicated in the development of multiple myeloma, breast and gastric cancer. These proteins interact with the centrosome and with INI probably through their interaction with Gas41.

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Abbreviations: DAPI: 4′,6-diamidino-2-phenyl indole, DMSO: dimethyl sulfoxide, EGTA: ethylene glycol tetraacetic acid, PMSF: phenyl methane sulfonyl fluoride.

However, in spite of these observations, it is still unclear to what extent the function of these proteins is evolutionarily conserved and whether Gas41 retains in mammals the role that Yaf9 seems to have in yeast. If this is the case, yeast would be further confirmed as an interesting model to investigate Gas41 role in the emergence of brain tumours and possibly their degree of malignancy.

In the present work we searched for possible interactors for Gas41 and demonstrated that it can interact with n-Myc and c-Myc proteins; the possible relationship between Gas41 and n-Myc expression was investigated in brain tumours. Finally, we proved that in yeast Gas41 N-terminal portion can functionally substitute for its Yaf9 counterpart.

MATERIALS AND METHODS

Peptides and antibodies. Recombinant Gas41 protein T7-tagged at N-terminus was from Abcam (Cambridge, UK: ab40052). Recombinant c-Myc (64 kDa) was from Active Motif (Rixensart, BE: 31117). Synthetic n-Myc peptide derived from residues 1–100 of human n-Myc was from Abcam (ab31595). Primary Antibodies: c-Myc (c33) mouse monoclonal antibody for detection of c-Myc p67 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal to n-Myc (ab16898), chicken polyclonal to Gas41 (ab15859) and goat polyclonal anti-T7 tag-agarose (ab1230) were from Abcam.

Cell cultures. Human neuroblastoma cell lines SK-N-SH and IMR32 (ATCC) and normal human fibroblasts MRC-5 (ATCC) were cultured in Minimum Essential Medium (MEM) and Dulbecco's modified Minimum Essential Medium (DMEM) supplemented with 1% Non Essential Amino Acids (NEAA), 1% glutamine (200 mM), sodium pyruvate (110 mg/ml), 10% fetal calf serum (FCS) (Sigma), penicillin (5000 IU/ml) and streptomycin (5 mg/ml).

Cell extract preparation. Whole cell extracts, cytoplasmic fractions and nuclear fractions were prepared with Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA: 40010) according to the manufacturer's instructions. The protein content of the fractions was quantified by Bradford method (Bradford 1976).

Construction of hybrid proteins. The *gas41* coding sequence was amplified by PCR from pBKCMV-gas41 (kind gift of U. Fischer) and cloned into vector pUG35. The primers used (Table 1) introduced a *Bam*HI site at the 5' end (G5B) and an in-frame *Sal*I site at the 3' end (G3S). The resulting vector contained a *gas41::GFP* fusion gene regulated by the *MET25* promoter. The following gene sequences were amplified with the indicated pairs of primers: *gas41* = 2HP1 and 2HP2; *gas41* N-half = 2HP1 and 2HP3; *gas41* C-half = 2HP4 and 2HP2; *YAF9* = 2HP5 and 2HP6; *YAF9* N-half = 2HP5 and 2HP7; *YAF9* C-half = 2HP8 and 2HP6. The oligonucleotides used as primers introduced *Sma*I (2HP1, 2HP5), *Pst*I (2HP2, 2HP6) or *Sal*I (2HP3, 2HP4, 2HP7 and 2HP8) sites. The fragments amplified were cut with the corresponding enzymes and inserted into pGBKT7 plasmid (Bittner *et al.,* 2004). The hybrid protein composed of the N-terminal half of Gas41 fused to the C-terminal half of Yaf9 was also cloned in the pESC-URA vector. To this purpose, the oligonucleotides used as primers introduced *Bam*HI (GY5B) and *Ava*I sites (GY3A).

Table 1. Primer list

Name	Sequence
G5B	CGGGATCCATGTTCAAGAGAATGGCCGA
G3S	ACGCGTCGACTATGTCTTTTGCTTGGTC
2HP1	TCCCCCGGGGATGTTCAAGAGAATGGCCGA
2HP2	AAACTGCAGTTATATGTCTTTTGCTTGG
2HP3	CGCGTCGACAAACAGCTTTAGCAAATGA
2HP4	CGCGTCGACCAATCAGACACCAATGCAA
2HP5	TCCCCCGGGGATGGCTCCGACAATAAGCA
2HP6	AAACTGCAGCTAACTTCCGTTAATGGC
2HP7	CGCGTCGACAGCATAAGGATGAAGTCG
2HP8	CGCGTCGACGCTAATCCTGTACCGAAT
GY5B	CGCGGATCCATGTTCAAGAGAATGGCCG
GY3A	TCCCTCGAGACTTCCGTTAATGGCTTC

Yeast two-hybrid analysis. The Matchmaker yeast two-hybrid system and the pre-transformed human foetal brain cDNA library were obtained from Clontech Laboratories (Palo Alto, CA, USA). After constructing pGBKT7-GAS41, the bait plasmid was transformed into yeast strain AH109 using the lithium acetate method and checked for absence of autoactivation or non-specific interactions between Gas41 and the GAL4 activation domain. Diploids that activated the *HIS3, ADE2* and *LacZ* reporter genes only in the presence of the pGBKT7-gas41 plasmid were considered positive. The pACT2 plasmids containing cDNAs encoding potential Gas41 interacting proteins were isolated as suggested by the supplier (Clontech Laboratories; Yeast Protocols Handbook PT3024-1), transformed into *Escherichia coli* cells (strain DH5-a; Invitrogen, Carlsbad, CA, USA) and sequenced.

Coupling of Gas41 agarose beads. Eight µl of recombinant T7-tagged Gas41 protein (0.5 µg/µl) was combined with 30 µl of anti-T7 tag agarose beads previously equilibrated in buffer G (20 mM Hepes pH 7.5, 100 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM EGTA, protease inhibitors cocktail). The mixture was kept at 4°C with mild agitation for 3 h. The beads were subsequently pelleted at 2000 g and the supernatant removed (FT, flow through). The beads were washed 4 times with 1 volume of buffer G and each time pelleted at 2000 g. No protein leakage was observed and the pelleted beads were immediately used for pull-down experiments.

Pull-down with recombinant c-Myc or n-Myc. Thirty µl of anti-T7 tag beads coupled with T7-tagged Gas41 was mixed with 30 µl of buffer G containing recombinant c-Myc or n-Myc peptides (ranging from 4 to 10 µg) and kept for 20 min at 4°C with mild agitation. The beads were subsequently pelleted and the supernatant removed (FT). The beads were washed 4 times and supernatants stored (W1-W4). The bound protein was eluted with 30 µl of sample buffer (62.5 mM Tris/HCL, pH 6.8, 2% SDS, 10% glycerol). As a control the same experiment was performed with 30 µl of anti-T7 tag-agarose not coupled to Gas41.

Pull-down cellular fractions. Thirty µg of IMR32 nuclear or cytoplasmic fraction, prepared as described, was mixed with 30 µl of anti-T7 tag beads coupled with T7-tagged Gas41. The beads were subsequently pelleted and the supernatant removed (FT). The beads were

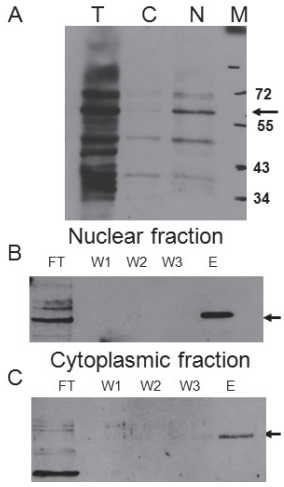

Figure 1. Pull-down experiments confirm Gas41-n-Myc interaction.
(A) Western blot of IMR32 total cell extract (T); cytoplasmic (C) and nuclear (F) fractions, and marker (M). **(B and C)** samples of supernatant of flow through (FT); wash (W1-W3) and elution (E) of pull down experiments performed with nuclear **(B)** or cytoplasmic **(C)** fractions on beads coupled to Gas41. Fractions (half volume each) were analysed by western blot. The arrows indicate unphosphorylated n-Myc (64 kDa).

washed 3 times and supernatants stored (W1-W3). The bound proteins were eluted with 30 µl of sample buffer. As a control the same experiment was performed with 30 µl of anti-T7 tag beads without Gas41.

Western blotting. Total proteins were extracted from tumour cell lines by RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP40, 1 mM PMSF, 1x protease inhibitor cocktail, Sigma), run in 12% SDS–PAGE and blotted on nitrocellulose membrane ProteanBA85 (Schleicher & Schuell/Whatman, Maidstone, Kent, UK). Membranes were incubated with anti-Gas41, anti-c-Myc or anti-n-Myc antibodies. The secondary HRP-conjugated antibodies used for the detection were rabbit polyclonal anti-chicken IgY (Abcam) and rabbit polyclonal anti-mouse IgG (Abcam), respectively. The enzymatic activity was revealed using ECL-detection kit (Pierce Biotechnology, Rockford, IL, USA). Anti-actin antibody (Chemicon/Millipore, Temecula, CA, USA) was used as reference.

***gas41* and n-*myc* expression.** Total RNA isolated from biopsies of 13 human brain tumours (5 glioblastomas, 4 anaplastic astrocytomas, 4 tumours with unclear histology) were analyzed using the AFFY_HG_U133_PLUS_2 Affymetrix microarrays. Expression levels of *gas41* (218911_at probeset) and n-*myc* (209756_s_at and 211377_x_at probesets) genes were calculated from complete raw data set after a standard GC-RMA pre-processing. The correlation analysis was performed with average values extracted from two independent experiments.

RESULTS

Identification of Gas41 interactors

The function of Gas41 could be clarified by the identification of physically interacting proteins. We therefore performed screening of a human foetal-brain cDNA library in yeast with the two-hybrid method, using Gas41 as bait. We constructed the Gas41 bait fused with the *GAL4* DNA-BD and we transformed a suitable mating partner (strain AH109, MAT a). The human foetal brain cDNA library, expressing proteins fused with the GAL4-AD, was provided in the yeast strain Y187 (MAT α). The two transformed cultures were mated to each other and diploid cells were selected. By plating the diploids on selective medium, only those containing putative interacting proteins could express the selectable marker gene(s) and grow. About 3×10^6 diploid clones were analysed in two independent screenings. Positive clones were tested also for interaction with Yaf9 (Table 2). Six proteins were confirmed as true interactors of Gas41: the cytoskeletal protein FRMD4A (two isolates) and the ubiquitination-related protein Rnf8 (one isolate), which both interacted also with Yaf9. Six isolates contained the oncogenic transcription factor n-Myc and interaction with Yaf9 was clone-dependent, probably because some of the isolates contained incomplete cDNA. Lsd1 demethylase (two isolates), a protein involved in chromatin modification, did not interact with Yaf9. One isolate contained ORF70 (HS chr17), whose interaction with Yaf9 was not tested.

Table 2. Gas41 interactors

Protein	Number of isolates	Interaction with Yaf9[1]
n-Myc	6	Clone dependent[2]
Lsd1	2	-
FRMD4A	2	+
Rnf8	1	+++
Hs Chr 17 ORF70	1	Not tested

[1]+++ = strong interaction; + = weak interaction; - = not interacting; [2]from strong to weak.

Validation of Gas41/n-Myc interaction in human cell lines

Among the identified Gas41 interactors we decided to further analyze Myc, because of its well known role in cell proliferation and its amplification in tumours, e.g., neuroblastomas. IMR32 neuroblastoma cell line showed

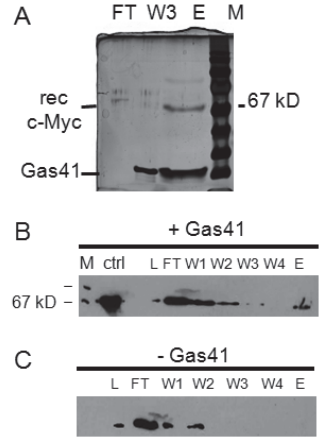

Figure 2. Purified recombinant c-Myc interacts with Gas41 *in vitro*.
(A) Silver stained protein gel (12% SDS/PAGE) showing a sample of supernatant of indicated fractions (FT: flow through; W3: third wash; E: eluate) of a pull-down experiment performed with recombinant c-Myc on beads coupled to Gas41. Half volume of each fraction was analysed. Position of Gas41 and recombinant c-Myc is indicated. **(B)** Western blot of a protein gel carrying indicated fractions (L: loaded sample; FT: flow through; W1-W4: washes; E: eluate) of a pull-down experiment performed with recombinant c-Myc on beads coupled to Gas41. 1/50th of volume of the load and 1/20th of each fraction were analysed. M=MW marker, ctrl=c-Myc. **(C)** Same experiment as in Fig. 3B, but performed with uncoupled beads.

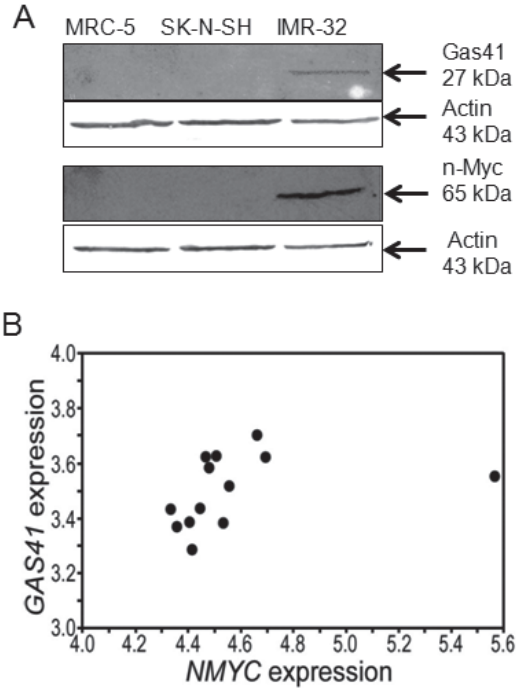

Figure 3. Gas41 and n-Myc show similar expression levels in brain tumours.
(A) Western blot analysis of MRC-5, SK-N-SH and IMR32 protein lysates. Arrows indicate Gas41 (27 kDa), n-Myc (64 kDa) and actin (43 kDa) signals. **(B)** Expression levels of *gas41* and n-*myc* genes were extracted from complete microrray analysis (Affymetrix) of tissue biopsies from 13 human brain tumours. Each dot represents expression levels for each tumour sample (log_2 expression signals).

a very high expression level of n-Myc (Schwab *et al.,* 1984) and therefore appeared an ideal system for validating its interaction with Gas41 by biochemical assays. Cytoplasmic and nuclear fractions were used for pull-down experiments with agarose beads coupled with Gas41. Figure 1A shows that both total cell extract and nuclear fraction contained a major n-Myc band and smaller (probably proteolytic) fragments. The nuclear extract showed two additional larger bands which could be nuclear phosphorylated forms. Both in the nuclear and in the cytoplasmic fraction (Fig. 1B and 1C), n-Myc seemed to bind to the Gas41-coupled beads mainly in the unphosphorylated form. No binding was observed with beads not coupled to Gas41 (not shown).

Since c-Myc can interact with the Tip60 complex through the Trrap protein component (McMahon *et al.,* 2000), we could not exclude that Trrap present in the extracts might mediate n-Myc binding to Gas41. To rule out this hypothesis we performed pull-down experiments using a purified recombinant c-Myc. We could detect c-Myc binding both by silver staining (Fig. 2A) and by western blotting with an antibody specifically recognizing c-Myc (Fig. 2B). When performing the pull-down with beads uncoupled to Gas41 (Fig. 2C), we did not detect c-Myc binding. This experiment demonstrates a direct interaction between c-Myc and Gas41. Since c-Myc and n-Myc share extensive homology at the C-terminal part, it is likely that interaction of both proteins with Gas41 is mediated by this portion. This hypothesis was confirmed by the absence of binding when pull-down was performed with 100 aminoacids of the N-terminal portion of n-Myc (synthetic peptide; data not shown).

Gas41 and n-Myc expression in brain tumours

We tested whether n-Myc and Gas41 were expressed at comparable levels in tumour cell lines. We analysed Gas41 and n-Myc expression in human neuroblastoma cell lines SK-N-SH and IMR32. In SK-N-SH cells, n-*myc* is not significantly amplified and/or expressed (Schwab *et al.,* 1984). It has been estimated that in IMR32 cells n-*myc* is amplified 15–20-fold and has 60-80-fold enhanced protein expression, as compared with SK-N-SH. We performed western blotting on protein extracts from cell lines SK-N-SH and IMR32, using anti-n-Myc monoclonal antibody and anti-Gas41 polyclonal antibody to detect protein levels. n-Myc and Gas41 signals appeared only in IMR32 neuroblastoma cell line (Fig. 3A). Moreover, n-*myc* RNA is 5–20-fold higher in IMR32 than in SK-N-SH (not shown). These results suggested that the over-expression of *gas41* and n-*myc* could be correlated events. In order to support this hypothesis we performed trascriptome analysis of biopsies from 13 human brain tumours (5 glioblastomas, 4 anaplastic astrocytomas, 4 tumours with unclear histology). A positive correlation (r=0.31, *p*-value=0.001) between the expression level of the two proteins was found (Fig. 3B). In order to test if there was a direct effect of Gas41 over-expression on n-Myc over-expression or vice versa, we transfected NIH-H1299 cells with FLAG-tagged Gas41 or with FLAG-tagged n-Myc and analyzed protein and RNA levels. No clear effect was observed, excluding a direct reciprocal regulation (not shown).

Table 3. Yaf9 and Gas41 vectors.

Protein[1]	Vector[2]	Promoter	Phenotypes[3]		
			DMSO	CsCl	Benomyl
Yaf9 (entire)	pUG35 (GFP)	MET25	+	+	+
Gas41 (entire)	"	"	-	-	-
Yaf9 (entire)	pGBT9 (DNA-BD)	tr*ADH1*[4]	+	-	-
Gas41 (N)+Yaf9 (C)	"	"	+	-	-
Yaf9 (N)+GAS41 (C)	"	"	-	-	-
Gas41 (entire)	"	"	-	-	-
Gas41 (N)+Yaf9 (C)	pESC-URA	GAL1	+	+	+
Yaf9 (entire)[5]	pGBKT7 (DNA-BD, c-myc)	ADH1	+	+	+
Yaf9 (N) only	"	"	-	-	-
Yaf9 (C) only	"	"	-	-	-

[1]N-half (N) and C-half (C) of Yaf9 protein were aminoacids 1 to 121 and 116 to 226, respectively. N-half (N) and C-half (C) of Gas41 were 1 to 121 and 122 to 227, respectively; [2]In parentheses the genetic elements fused to the gene sequence are indicated; [3]+ and – indicate growth and non-growth on the indicated xenobiotic; [4]tr*ADH1* is a truncated form of the *ADH1* promoter with reduced transcription activity; [5]This set of vectors was kindly provided by R. Slany (Bittner *et al.,* 2004).

Expression of Gas41 in *S. cerevisiae*

Gas41 and Yaf9 share significant sequence homology (Le Masson *et al.,* 2003) but

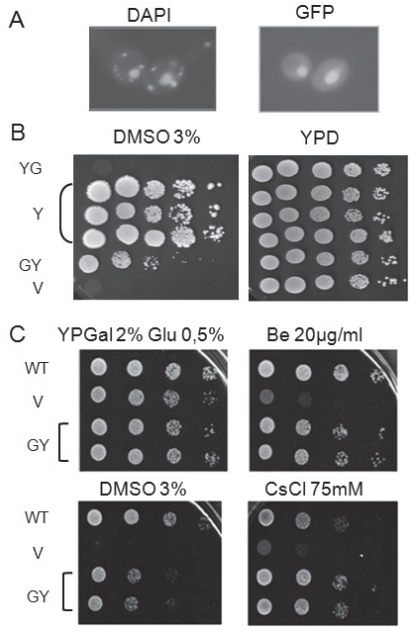

Figure 4. Gas41 and Yaf9 amino-terminal portions are interchangeable.
(A) Nuclear localization in yeast of human Gas41::GFP protein (right). Cell stained with DAPI (left) shows nuclei positioning. **(B)** Phenotypic suppression of DMSO sensitivity (left) of *yaf9*Δ strain transformed with hybrid proteins. Gas41 N-half and Yaf9 C-half: row GY. Reciprocal hybrid protein: row YG. Positive (three independent clones) and negative controls: rows Y (entire Yaf9) and V (vector), respectively. Growth on medium (YPD) without inhibitor is shown at right. **(C)** Suppression by hybrid protein (two clones, rows GY) of sensitivity to Benomyl (upper-right), DMSO (lower-left) and CsCl (lower-right) of *yaf9*Δ strain. Controls are wild-type (row WT) strain and *yaf9*Δ strain transformed with the empty vector (V). Growth on medium (YPGal-Glu) without inhibitors is reported in the upper-left.

it is still unclear to what extent they can be considered functional homologues. We assayed a functional substitution of Yaf9 with Gas41 in yeast by cloning *gas41* cDNA, kindly provided by U. Fischer, into the centromeric yeast vector pUG35 and generating a fusion protein with GFP. After transformation of the *yaf9*Δ strain, we observed by fluorescent microscopy that Gas41 was expressed and was localized in the nucleus (Fig. 4A), similarly to what was previously observed with a Yaf9-GFP fusion (Le Masson *et al.,* 2003). The *yaf9*Δ strain is sensitive to CsCl, DMSO and Benomyl (Bianchi *et al.,* 2004): however, the expression of Gas41 did not suppress any of the *yaf9*Δ strain phenotypes (Table 3) suggesting that Gas41 was lacking functionality of domain(s) required for biological functions in yeast. To identify these domains, we constructed Gas41-Yaf9 hybrid proteins having exchanged N-halves and C-halves, as described in Table 3, and we tested them for phenotype suppression. The chimeric genes were cloned in vectors pGBT9 and pESC-URA and used to transform *yaf9*Δ strain. The presence of the expected proteins in the transformed clones was verified by western blotting (not shown). Results of suppression (Fig. 4B and 4C) clearly showed that only the chimeric protein composed of the N-terminal half of Gas41 and the C-terminal half of Yaf9 was able to restore growth on inhibitors. Results also suggested that the level of gene expression, *i.e.* strength of the promoter, might specifically influence the resistance to individual inhibitors. Control experiments showed that expression of only the N-terminal half or the C-terminal half of Yaf9 was unable to suppress sensitivity to inhibitors (Table 3). These findings indicated that the N-terminal halves of Gas41 and Yaf9, whose protein sequences are more conserved than the C-terminal halves (Le Masson *et al.,* 2003), were functionally exchangeable. The inability of the C-terminal half of Gas41 to restore growth and the sequence divergence of Gas41 and Yaf9 in these portions suggested that species specific functions might rely on the C-terminal half.

DISCUSSION

We have identified six new Gas41 molecular interactors, including two proteins involved in regulation of gene expression: Lsd1 and n-Myc. We focused our attention to the latter and performed experiments to confirm the interaction between Gas41 and n-Myc, while the interactions between Gas41 and the other five proteins will require further *in vivo* and/or *in vitro* investigations. n-Myc is a member of a family of oncoproteins involved in transcription regulation and targeting of chromatin modification complexes at active promoters (Patel *et al.,* 2004; Martinato *et al.,* 2008; Perini *et al.,* 2005). In our two-hybrid screening with Gas41, we isolated six clones containing different n-Myc portions. Interaction with Yaf9 was clone-dependent, suggesting that different domains of n-Myc could be involved in the interaction; however, *in vitro* binding of Gas41 with the N-terminal part of n-Myc did not occur. Our results indicate that also c-Myc can directly interact with Gas41. It has been shown that the HAT-containing Tip60 complex is targeted to active promoters by c-Myc through an interaction with the Trrap subunit (McMahon *et al.,* 2000; Martinato *et al.,* 2008; Frank *et al.,* 2003) involving the N-terminal portion of c-Myc (McMahon *et al.,* 2000). Since c-Myc and n-Myc share extensive homology at the C-terminus, our results suggest a second targeting mode, mediated by Gas41, which could be used by Myc proteins to recruit chromatin modification machineries. The interaction between Myc proteins and Gas41 might also be confirmed by additional approaches, such as reverse pull-down experiments.

In addition to their physical interaction, a correlation between Gas41 and n-Myc is also suggested by their overexpression in tumour cell line IMR32 and in several brain tumours, independently of malignancy degree. However, although the P-value associated with the co-expression of Gas41 and n-Myc is low ($P < 0.001$), the detail of the functional interaction between the two proteins remains to be demonstrated. Moreover, it does not seem to derive from a direct reciprocal regulatory control of the two genes, as suggested by experiments in NIH-H1299 cells. We can only speculate that n-*myc* amplification in brain tumours might select *gas41* amplifying or over-expressing cells which can better exploit the augmented targeting capability of n-Myc. The crucial importance of this recruiting activity for transcription regulation is probably at the base of the evolution of multiple interactions between the Myc proteins and Tip60 and SCRAP complex subunits. It has been proposed that Gas41 (Doyon & Cote 2004), as its yeast counterpart Yaf9, is associated with other three subunits (Dmap1, Baf53 and actin) in a recruiting module distinct from the one containing Trrap. It is possible that the two different modules have different interaction partners and different influence on the targeting of Tip60 and SCRAP.

Lsd1 is a FAD-dependent amine oxidase which promotes histone demethylation and which has been found in complexes commonly involved in repression of transcription. However, Lsd1 has been found to operate also

in transcription activation (Metzer & Schule 2007; Perillo *et al.,* 2008) and a model of cooperation between Lsd1 and Myc has been proposed for gene expression (Amente *et al.,* 2010). Although not yet demonstrated in human cells, we have found that Lsd1 specifically interacts with Gas41 in yeast, suggesting that the cooperation between Lsd1 and Myc might involve Gas41. Another protein interacting with Gas41 is the FERM domain-containing protein 4A. FRMD4A also interacts with Yaf9 and has a hypothetical function in cytoskeletal dynamics. The FERM domain is involved in protein-protein interactions and is present in several proteins, primarily involved in structural functions, but also in signaling (kinases and phosphatases) and, interestingly, in Merlin, the neurofibromastosis 2 tumour suppressor protein (Gusella *et al.,* 1999).

We also demonstrate here that Gas41 N-terminal half can substitute in yeast for the corresponding Yaf9 portion and suppress sensitivity phenotypes associated with transcriptional regulation and genetic instability observed in *yaf9*Δ strains (Le Masson *et al.,* 2003; Bianchi *et al.,* 2001; 2004). The N-terminal half of these proteins is conserved and contains the YEATS domain which is likely to have conserved functions in different biological systems. The YEATS domain of ENL protein binds histones H3 and H1 (Zeisig *et al.,* 2005) and is involved in targeting chromatin-modifying complexes (Schultze *et al.,* 2009). The C-terminal half of Gas41, which does not substitute for the C-terminal of Yaf9 in phenotypic suppression and contains a coil-coiled domain required for its incorporation in the SCRAP and Tip60 complexes (Park & Roeder 2006), might have diverged during evolution, adapting to different protein-protein interactions with coevolving partners.

In conclusion, we have demonstrated that the evolutionarily conserved protein Gas41 can interact with proteins and/or complexes involved in transcription regulation by mechanisms that might be different from those proposed until now, suggesting a multivalent role of this protein in gene expression regulation. We have also found a common regulation of Gas41 and Myc in brain tumour-derived cell lines and biopsies. Finally, the demonstration of functional homology of Gas41 and Yaf9 N-terminal regions confirms that the use of yeast may help to identify the role of Gas41 in brain tumours.

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