

# MYCN repression of Lifeguard/FAIM2 enhances neuroblastoma aggressiveness

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Neuroblastoma (NBL) is the most common solid tumor in infants and accounts for 15% of all pediatric cancer deaths. Several risk factors predict NBL outcome: age at the time of diagnosis, stage, chromosome alterations and MYCN (V-Myc Avian Myelocytomatosis Viral Oncogene Neuroblastoma-Derived Homolog) amplification, which characterizes the subset of the most aggressive NBLs with an overall survival below 30%. MYCN-amplified tumors develop exceptional chemoresistance and metastatic capacity. These properties have been linked to defects in the apoptotic machinery, either by silencing components of the extrinsic apoptotic pathway (e.g. caspase-8) or by overexpression of antiapoptotic regulators (e.g. Bcl-2, Mcl-1 or FLIP). Very little is known on the implication of death receptors and their antagonists in NBL. In this work, the expression levels of several death receptor antagonists were analyzed in multiple human NBL data sets. We report that Lifeguard (LFG/FAIM2 (Fas apoptosis inhibitory molecule 2)/NMP35) is downregulated in the most aggressive and undifferentiated tumors. Intriguingly, although LFG has been initially characterized as an antiapoptotic protein, we have found a new association with NBL differentiation. Moreover, LFG repression resulted in reduced cell adhesion, increased sphere growth and enhanced migration, thus conferring a higher metastatic capacity to NBL cells. Furthermore, LFG expression was found to be directly repressed by MYCN at the transcriptional level. Our data, which support a new functional role for a hitherto undiscovered MYCN target, provide a new link between MYCN overexpression and increased NBL metastatic properties.

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Neuroblastoma (NBL) is the most common solid tumor of infancy, accounting for 15% of all pediatric cancer deaths. These tumors are very heterogeneous, with a clinical course ranging from spontaneous regression to aggressive behavior. Several risk factors predict NBL outcome: INSS (International Neuroblastoma Staging System) tumor stage, age at diagnosis, INPC (International Neuroblastoma Pathology Classification) classification, DNA ploidy and MYCN (V-Myc Avian Myelocytomatosis Viral Oncogene Neuroblastoma-Derived Homolog) oncogene amplification, which characterizes the subset of most aggressive NBLs with overall survival below 30%.<sup>1–3</sup> MYCN-amplified tumors are characterized by exceptional chemoresistance and metastatic capacity. These properties have been linked to defects in the apoptotic arsenal, either by overexpression of the antiapoptotic regulators of the mitochondrial pathway (e.g. Bcl-2, Mcl-1)<sup>4,5</sup> or by alteration of components of the extrinsic apoptotic pathway (e.g. caspase-8).<sup>6–8</sup> In fact, there is evidence that the extrinsic

pathway may serve as a checkpoint to guard cells from MYCN-initiated tumorigenesis as MYCN-elevated levels sensitize NBL cells to death receptor (DR)-induced cell death, either by TRAIL, TNF $\alpha$  or FasL stimuli.<sup>9</sup> To date, the main mechanism underlying the lack of DR-induced apoptosis in MYCN-amplified tumors has been methylation of the caspase-8 promoter, which blocks its expression and renders cells resistant to DR-induced cell death.<sup>7,10</sup> However, the correlation between MYCN amplification and caspase-8 silencing in tumor samples remains controversial; other authors showed the inactivation of caspase-8 to be independent of MYCN amplification and NBL prognosis.<sup>6</sup> As MYCN amplification and caspase-8 silencing may not occur simultaneously, alternative resistance mechanisms must exist, which either block DR-induced cell death or switch DR signaling to alternative functions. DR activity can also be modulated by DR antagonists, which have been poorly characterized in the context of NBLs. The present work sought to analyze the role

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**Abbreviations:** BLI, bioluminescence; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle's medium; DR, death receptor; EMT, epithelial-to-mesenchymal transition; FAIM2, Fas apoptosis inhibitory molecule 2; FBS, fetal bovine serum; GFP, green fluorescent protein; HDAC, histone deacetylase; INPC, International Neuroblastoma Pathology Classification; KEGG, Kyoto Encyclopedia of Genes and Genomes; LFG, Lifeguard; MYCN, V-Myc Avian Myelocytomatosis Viral Oncogene Neuroblastoma-Derived Homolog; NaB, sodium butyrate; NBL, neuroblastoma; PBS, phosphate-buffered saline; qPCR, quantitative real-time polymerase chain reaction; RA, retinoic acid; RPMI, Roswell Park Memorial Institute; Scr, scrambled; S.E.M., standard error of the mean; Sh, short hairpin; TJ, tight junction

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of DR antagonists in NBL and their contribution to the oncogenic properties of NBL cells. Several DR antagonists were found to be differentially expressed in the highest-risk NBL tumors, namely stage 4 MYCN-amplified NBLs. Among these, FAIM2 (Fas apoptosis inhibitory molecule 2), most commonly referred to as Lifeguard (LFG), correlates best with worse overall survival of NBL patients. Furthermore, we show that MYCN is able to repress directly *LFG* expression, which results in increased oncogenic properties such as augmented sphere formation, decreased adhesion and enhanced migration. Collectively, our results demonstrate a previously unappreciated role of LFG, and support a new target for therapeutic intervention against high-risk NBL.

## Results

**The DR antagonist LFG is downregulated in high-risk NBL.** The mRNA expression levels of antiapoptotic genes known to modulate the extrinsic pathway in the neural lineage were analyzed in independent human expression and prognostic NBL data sets. Few DR modulators were consistently altered in different NBL data sets. Table 1 shows the fold change variation between stage 4 MYCN-amplified tumors versus the rest of stages present in the respective study (i.e. stages 1, 3 and 4 without MYCN amplification). *PEA15*, *CFLAR/FLIP* and *LFG* were downregulated in advanced stages of the disease. In particular, the highest differential expression was found in stage 4 MYCN-amplified patients, the group of NBLs with the worst prognosis (Figure 1a). Whether the differences in gene expression could be associated with patient outcome was also analyzed. Notably, only low expression levels of *LFG* were associated with worse overall survival (Figure 1b and Table 1). Moreover, the association between *LFG* levels and patient survival was maintained in MYCN non-amplified tumor subsets (Supplementary Figure 1). These differences in *LFG* expression levels were then validated in an independent cohort of 38 primary tumor samples obtained at the Vall d'Hebron Hospital (Supplementary Table 1). Quantitative real-time PCR (qPCR) expression analysis revealed a

significant reduction in *LFG* levels in stage 4 and MYCN-amplified tumors (Figure 1c). Western blot analyses confirmed that differences in mRNA expression were also found at the protein level (Figure 1d). In summary, we conclude that LFG is the DR antagonist with the highest differential expression and association with patient outcome in high-risk NBL.

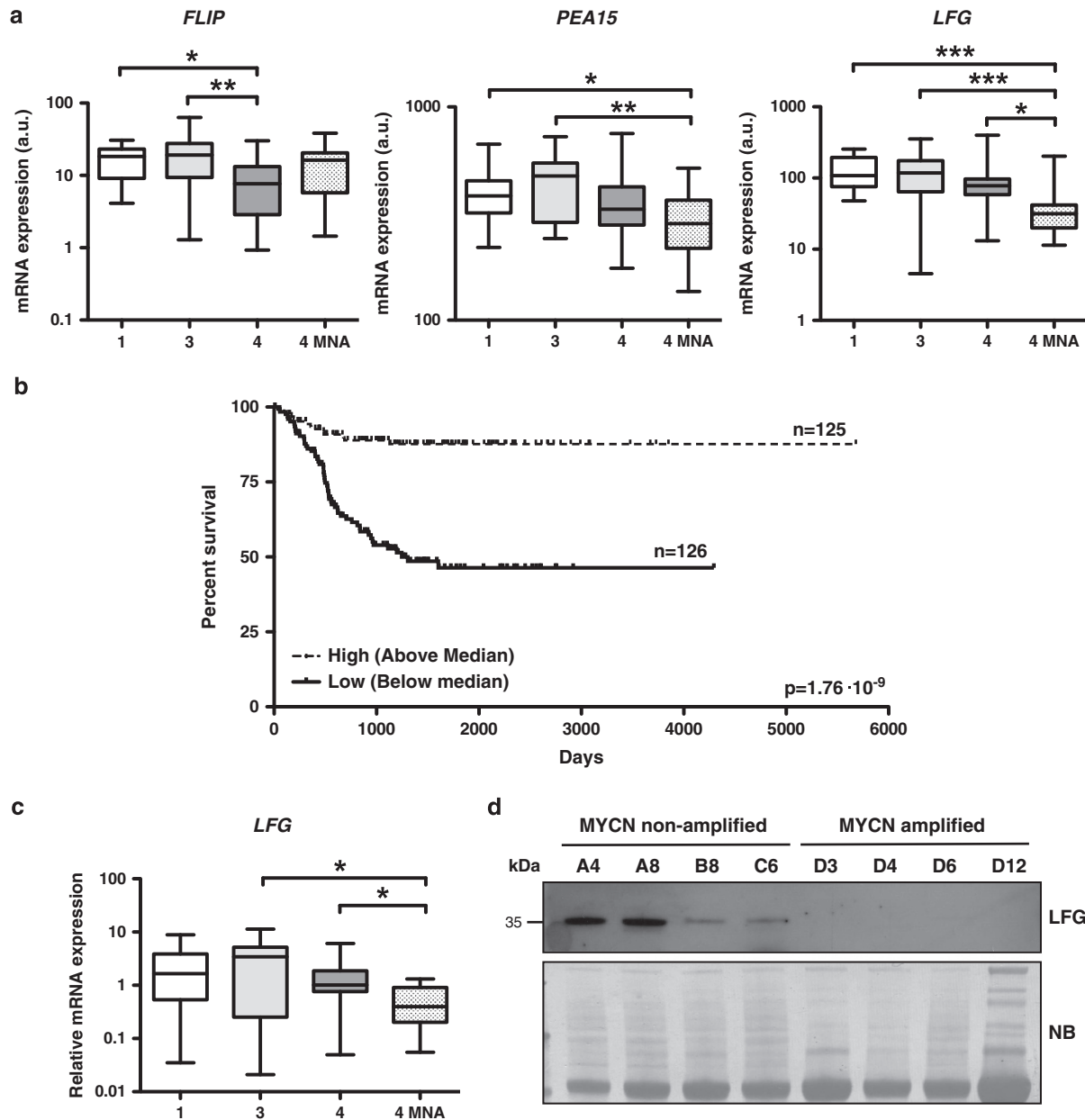
**LFG is associated with NBL differentiation.** When *LFG* levels were correlated with a number of clinicopathologic variables (i.e. age at diagnosis, site of primary tumor, site of metastasis and histology), *LFG* expression was found to be closely associated with tumors presenting unfavorable histology ( $P=0.0041$ ) and MYCN amplification ( $P=0.0121$ ), which are often undifferentiated tumors<sup>11</sup> (Figure 2a and Supplementary Table 2). With a view to ascertain the association between tumor differentiation and *LFG* expression, we sought to characterize *LFG* levels in the different status of NBL cell differentiation. On the one hand, *all-trans* retinoic acid (RA)-mediated differentiation of NBL cell lines increased *LFG* at mRNA and protein levels (Figures 2b and c). *TRKB* was used as a neuronal differentiation marker<sup>12</sup> to confirm the differentiation response of the NBL cell lines to the RA treatment. On the other hand, *LFG* levels were analyzed in cells cultured in neurosphere formation medium (serum-free and supplemented with EGF/FGF2), which mimics undifferentiated status of NBL cells.<sup>13,14</sup> When NBL cell lines were seeded in neurosphere medium, cells formed non-adherent spheres. *LFG* was found to be downregulated in sphere medium-cultured cells compared with cells cultured in standard conditions ( $P=0.0286$  for SK-N-BE(2) and IMR-5;  $P=0.0294$  for IMR-32) (Figure 2d). We conclude that *LFG* expression associates with the differentiation status of NBL cells.

**Reduction of *LFG* levels modulates proliferation, adhesion and migration of NBL cells.** To assess the role of LFG in NBL oncogenic properties, loss-of-function experiments were performed using two different short hairpin (sh)RNA targeting LFG (Figure 3a). Both sh*LFG* constructs

**Table 1** Death receptor antagonist expression and correlation with survival in neuroblastoma data sets

Gene symbol	Alias	Changes in expression levels		Correlation with survival	
		FC (P-value) GSE3960	FC (P-value) GSE16237	Worse if (P-value) GSE16476	Worse if (P-value) Oberthuer lab
<i>FAIM</i>	FAIM1	NA	1.00 (= 0.6430)	Low (= 0.419)	NA
<i>FAIM2</i>	Lifeguard	- 3.33 (< 0.0001)	- 3.22 (= 0.0002)	Low (= 0.019)	Low (= 1.76e <sup>-9</sup> )
<i>FAIM3</i>	TOSO	- 1.69 (= 0.2831)	1.00 (= 0.8994)	High (= 0.675)	NA
<i>CFLAR</i>	FLIP	- 1.56 (= 0.0154)	- 2.43 (= 0.0235)	Low (= 0.106)	Low (= 0.978)
<i>XIAP</i>	IAP3, BIRC4	1.57 (= 0.3988)	- 1.20 (= 0.0638)	Low (= 0.128)	NA
<i>BIRC3</i>	IAP2	- 3.57 (< 0.0001)	- 1.28 (= 0.2423)	Low (= 0.514)	Low (= 0.026)
<i>BIRC2</i>	IAP1	- 1.19 (= 0.0011)	- 1.49 (= 0.0954)	Low (= 7.01e <sup>-9</sup> )	Low (= 5.6e <sup>-4</sup> )
<i>TNFAIP3</i>	A20	- 2.27 (< 0.0001)	1.05 (= 0.2570)	Low (= 0.459)	Low (0.141)
<i>BFAR</i>	BAR, RNF47	NA	1.55 (= 0.2570)	High (= 0.001)	High (= 0.024)
<i>PTPN13</i>	FAP-1	1.36 (= 0.1322)	- 1.19 (= 0.7333)	High (= 0.24e <sup>-3</sup> )	NA
<i>PEA15</i>	HMAT1	- 1.33 (= 0.0154)	- 1.36 (= 0.0247)	Low (= 0.367)	NA
<i>SUMO1</i>	UBL1, PIC1	1.25 (= 0.0044)	- 2.27 (= 0.3990)	High (= 0.547)	High (= 0.031)
<i>BTK</i>	ATK, XLA	NA	- 1.20 (= 0.9880)	High (= 0.373)	Low (= 0.398)

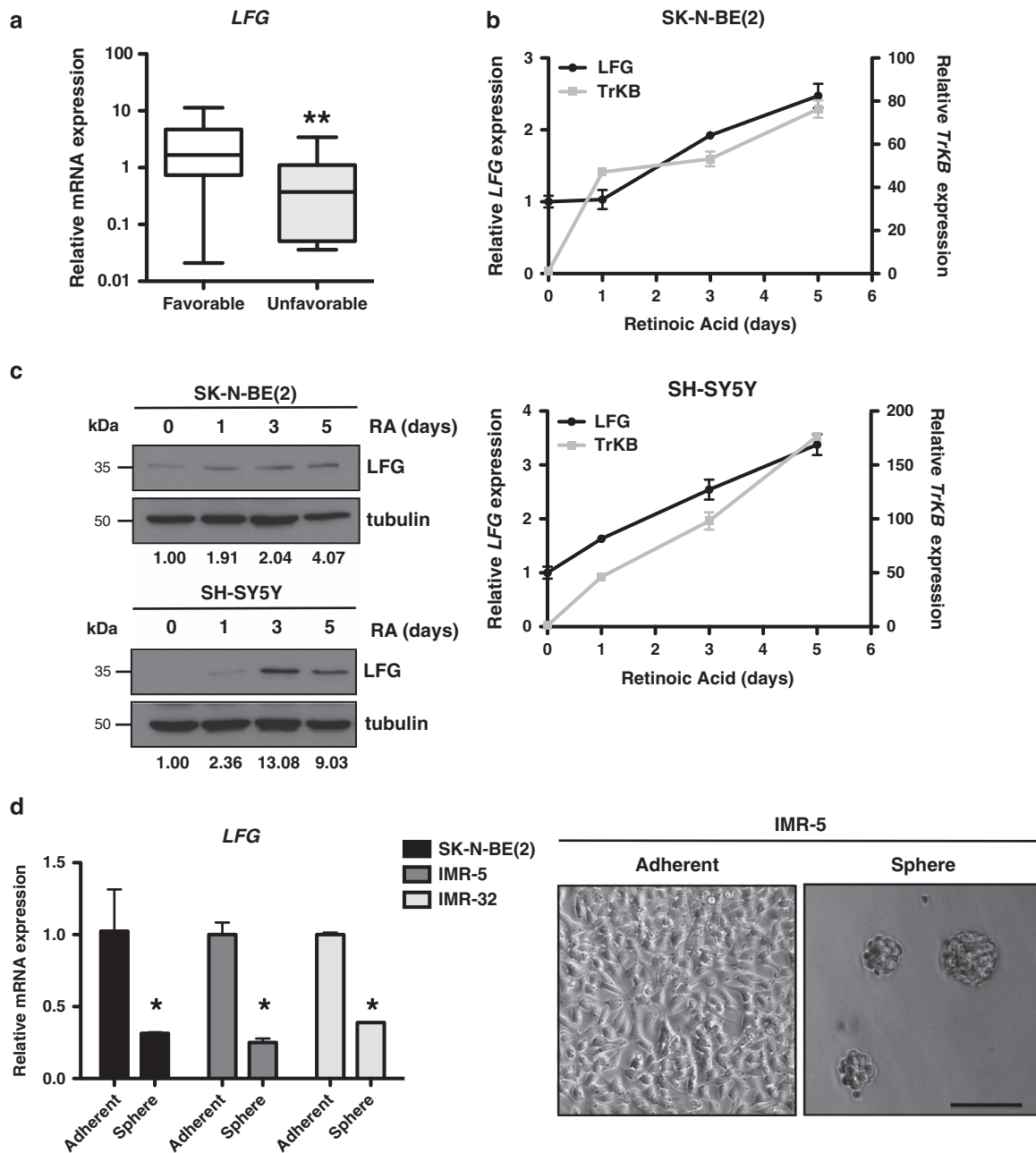
Abbreviations: FC, fold change; NA, not available  
Statistically significant P-values are highlighted in gray



**Figure 1** LFG is downregulated in high-risk NBL. (a) mRNA expression of the DR antagonists *FLIP* (1868\_g\_at), *PEA15* (32260\_at) and *LFG* (33293\_at) in different NBL stages obtained from GSE3960 data set. Data were analyzed by one-way ANOVA; \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . MNA, MYCN amplified. (b) Kaplan–Meier overall survival curve in 251 tumors based on high or low *LFG* expression (probe A\_23\_P139891). (c) Relative *LFG* expression measured by qPCR in an independent cohort of NBL primary tumors collected at Vall d’Hebron Hospital. Data were analyzed by one-way ANOVA; \* $P < 0.05$ . (d) Western blot showing *LFG* expression in MYCN non-amplified versus four MYCN-amplified primary NBL tumors. Naphtol blue (NB) staining was used as a loading control

notably knocked down LFG at the protein level and reduced proliferation of SH-SY5Y ( $P=0.022$  for sh*LFG* and  $P=0.0238$  for sh*LFG2*) and CHLA-90 cells ( $P=0.0043$  for sh*LFG* and  $P=0.022$  for sh*LFG2*) (Figure 3b). The most efficient downregulation was achieved by sh*LFG*, which was used for the following experiments. Cell adhesion assays showed that sh*LFG*-transduced cells exhibit around 50% reduction in adhesion to adherent plates compared with scrambled (Scr)-infected cells ( $P=0.0286$  for SH-SY5Y and  $P=0.0043$  for CHLA-90 cells) (Figure 3c). Moreover, when NBL cells were seeded at low density, loss of LFG increased

non-adherent growth compared with the Scr shRNA (Figure 3d). More than 10% of sh*LFG* cells were able to form viable spheres when they were seeded at low density in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% fetal bovine serum (FBS), whereas  $< 4\%$  of control cells did so ( $P=0.0066$ ). Furthermore, sh*LFG*-infected cells showed a fourfold increased migration through the 8  $\mu$ m-transwell polycarbonate inserts ( $P=0.0191$ ) (Figure 3e). The impact of these properties was further evaluated *in vivo*. SH-SY5Y cells (sh*LFG* or shScr) were injected into the flank of female NMRI-Foxn1nu/Foxn1nu mice. Tumors appeared

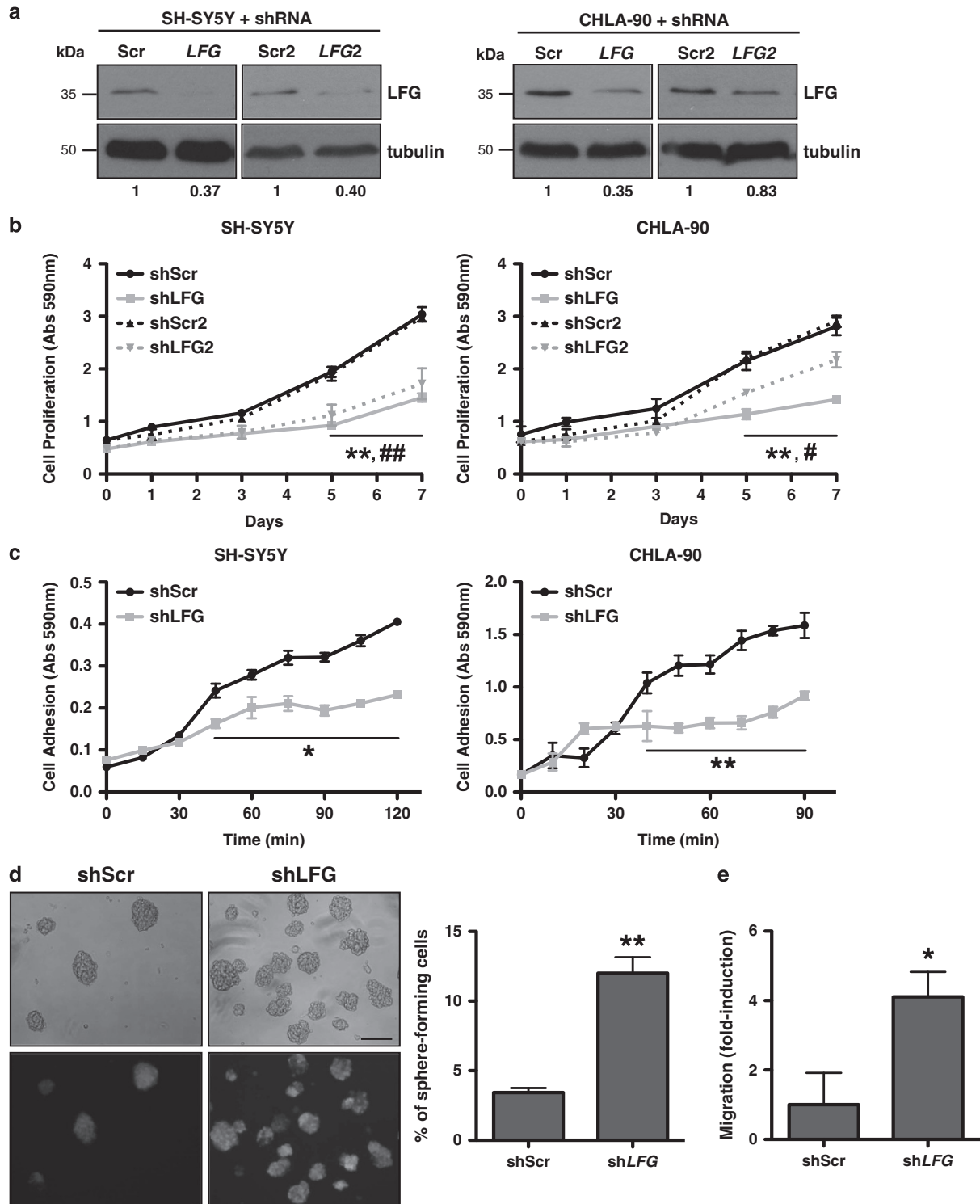


**Figure 2** *LFG* levels are associated with NBL differentiation. (a) Relative *LFG* expression in histologically favorable ( $n=30$ ) versus unfavorable ( $n=8$ ) primary tumors. mRNA levels were measured by qPCR.  $**P<0.01$ , unpaired  $t$ -test. (b) *All-trans* RA ( $10\ \mu\text{M}$ ) induced differentiation in NBL cell lines SK-N-BE(2) and SH-SY5Y. *LFG* and *TRKB* expression were measured by qPCR at the indicated time points. Data are presented as mean  $\pm$  S.E.M. of three independent experiments. (c) Western blot of *LFG* expression.  $\alpha$ -Tubulin was used as a loading control. Bands were quantified using Image J software (National Institutes of Health, Bethesda, MD, USA) and normalized expression values are indicated. (d) *LFG* expression detection by qPCR in the indicated NBL cell lines cultured with standard conditions (Adherent) or using neurosphere medium (Sphere).  $*P<0.05$ , unpaired  $t$ -test; data are represented as mean  $\pm$  S.E.M.,  $n=4$ . Images (right) show IMR-5 adherent and sphere-forming cultures. Scale bar,  $100\ \mu\text{m}$

earlier in the *LFG* knockdown group (Figure 4a). No significant differences in tumor growth or tumor weight were found (Figures 4b and c). The presence of spontaneous metastasis was evaluated in the lungs by *ex vivo* bioluminescence (BLI) detection. Mice inoculated with *LFG* knockdown cells showed increased metastatic burden in the lungs and higher number of metastatic lesions (Figures 4d and e). Taken together, these results indicate that the reduction on

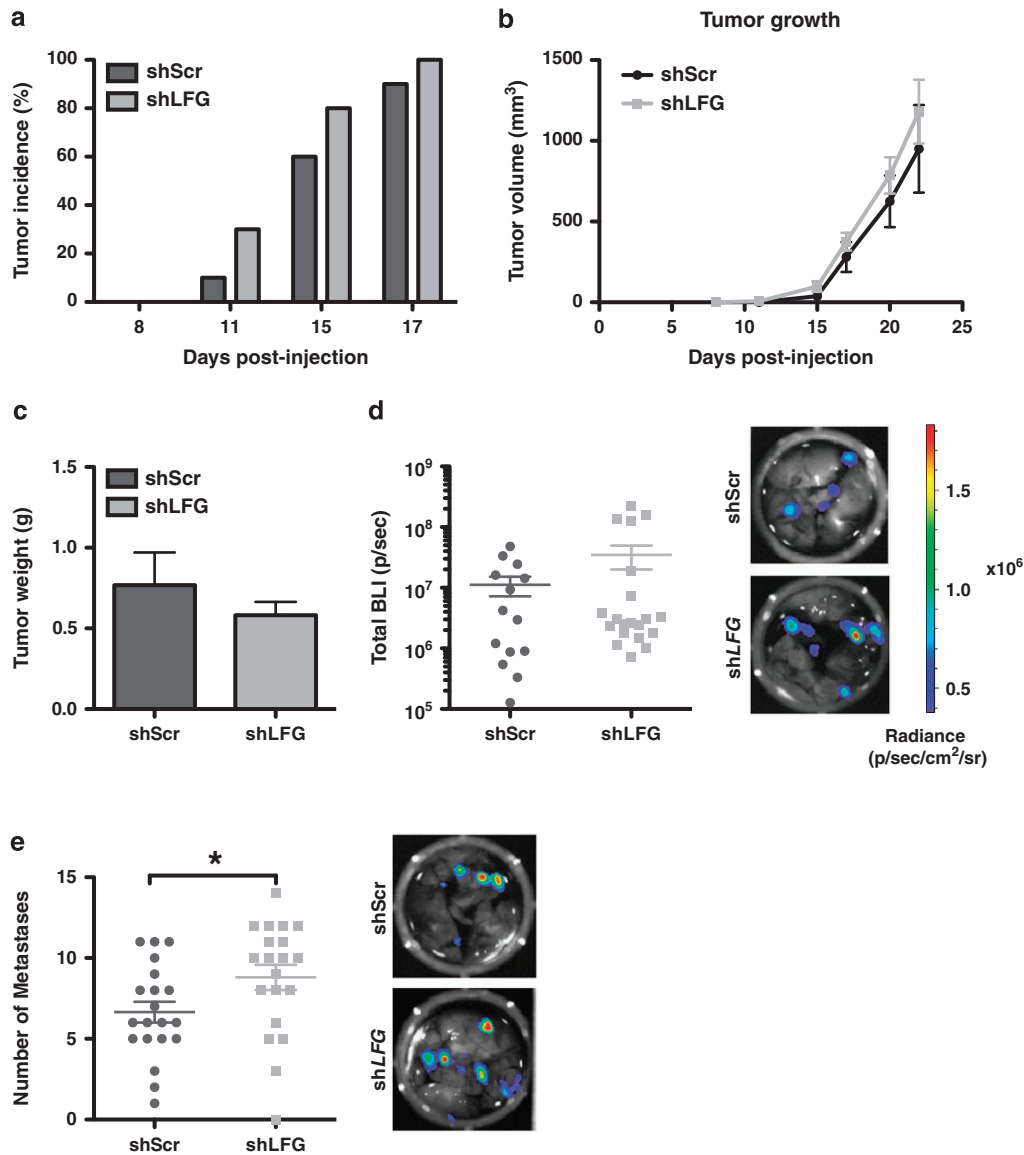
*LFG* levels may contribute to altered adhesion properties and increased metastatic potential of NBL cells.

***LFG* knockdown alters cell adhesion programs.** To enlighten the mechanism by which *LFG* knockdown reduces cell adhesion and enhances migration properties of NBL cells, we proceeded to *LFG* knockdown and performed a whole transcriptome analysis by Affymetrix expression



**Figure 3** LFG knockdown favors the metastatic potential of NBL cell lines. (a) Western blot showing LFG downregulation after infection with shScr or shLFG lentiviruses using anti-FAIM2 (H-21) antibody (Santa Cruz Biotechnology Inc). Bands were quantified and normalized versus  $\alpha$ -tubulin. (b) Cell proliferation assay in SH-SY5Y and CHLA-90 cells after LFG downregulation with two shRNA sets (shScr/shLFG and shScr2/shLFG2). Cells were fixed with 4% paraformaldehyde at the indicated time points. Data are presented as mean  $\pm$  S.E.M. of three independent experiments. \* $P < 0.05$  and \*\* $P < 0.01$ , unpaired *t*-test; #shScr versus shLFG; #shScr2 versus shLFG2. (c) Cell adhesion assay of SH-SY5Y and CHLA-90 transduced with either Scr or LFG shRNAs, measured at the indicated time points. \* $P < 0.05$  and \*\* $P < 0.01$ , unpaired *t*-test; data are represented as mean  $\pm$  S.E.M.,  $n = 3$ . (d) Sphere formation assay of shScr and shLFG SH-SY5Y cells seeded at low density in DMEM + 15% FBS and kept in standard culture conditions for 4 weeks. Graph represents the percentage of sphere formation. \*\* $P < 0.01$ , unpaired *t*-test; data are presented as mean  $\pm$  S.E.M.,  $n = 9$ . Infection efficiency was monitored by visualization of green fluorescent protein (GFP). Scale bar, 100  $\mu$ m. (e) Transwell migration assay of shScr and shLFG-transduced SH-SY5Y cells. Cells that migrated were quantified by WST-1 assay. \* $P < 0.05$ , unpaired *t*-test; graphs show mean  $\pm$  S.E.M.,  $n = 3$

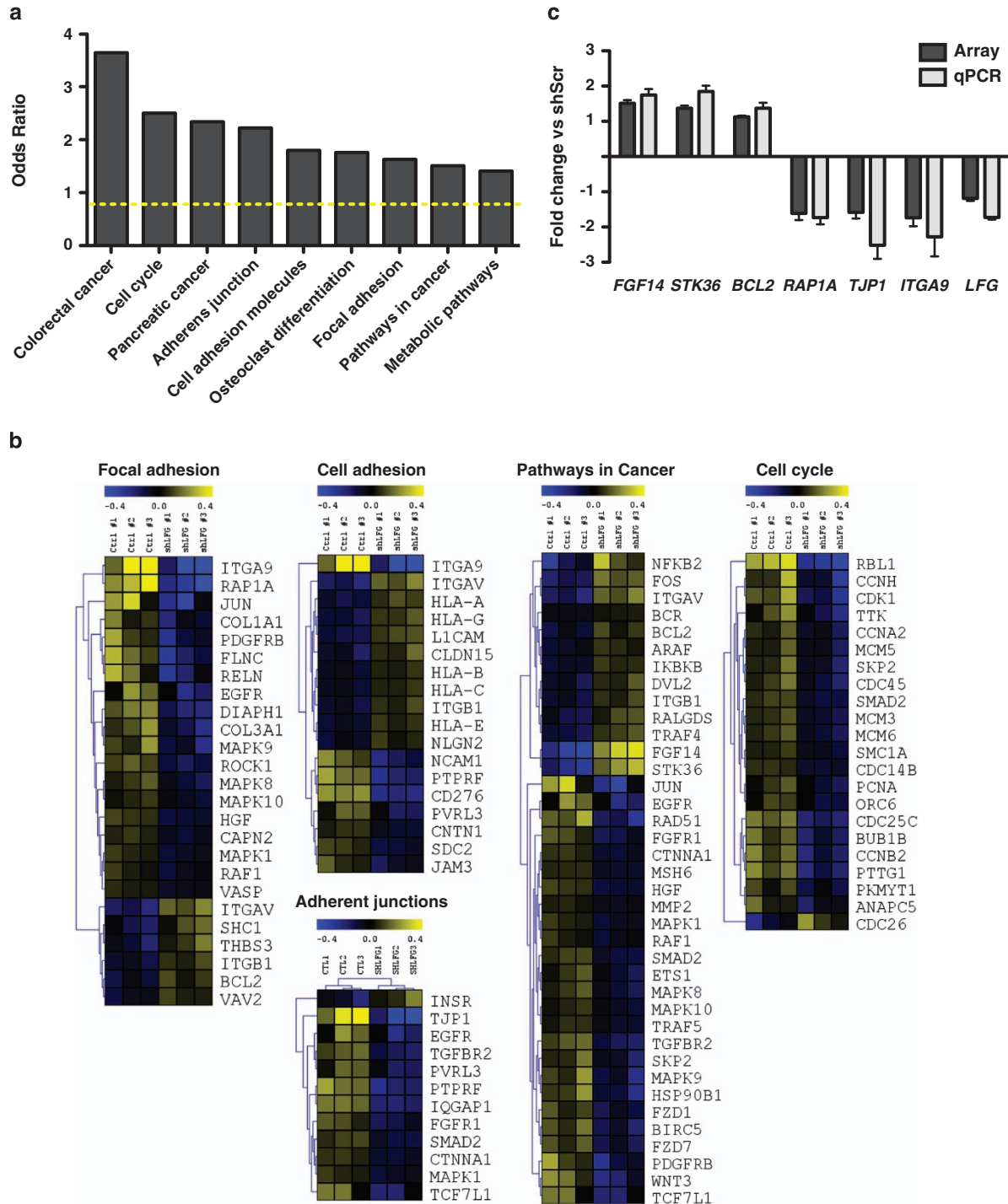




**Figure 4** *LFG* knockdown increases the metastatic potential of SH-SY5Y cells. (a) Xenograft formation assay with shScr and shLFG SH-SY5Y cells injected into the flank of NMRI nude mice. (b) Tumor volume measured at different days postinjection using a digital caliper. Data are presented as mean  $\pm$  S.E.M. (c) Tumor weight measured after surgery. Data are presented as mean  $\pm$  S.E.M. (d) Total BLI flux (photons/s) of lung metastases from shScr and shLFG SH-SY5Y-injected mice. Images are representative of *ex vivo* BLI lung images. Pictures were set at the same scale to compare BLI between shScr and shLFG conditions. (e) Scoring of lung metastases. Data are shown as mean  $\pm$  S.E.M. Images are representative of *ex vivo* BLI showing distant metastases in lungs dissected from shScr and shLFG-injected mice

arrays. Principal component analysis segregated the infected cells (Scr versus shLFG), indicating a consistent transcriptional impact of *LFG* knockdown (Supplementary Figure 2). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis<sup>15,16</sup> of genes differentially expressed in response to *LFG* knockdown revealed a reproducible modulation of genes mainly involved in cancer pathways and adhesion processes (Figure 5a). Functional annotation analysis showed a remarkable modulation of genes implicated in focal adhesion, cell adhesion and adherent junctions (Figure 5b), which supported an active role of *LFG* in the tumorigenic properties of NBL cells. Array data were validated by qPCR for *FGF14*, *STK36*, *BCL2*, *RAP1A*, *TJP1*, *ITGA9* and *LFG* (Figure 5c).

***LFG* expression is directly modulated by MYCN.** As *LFG* levels were significantly lower in MYCN-amplified tumors, we sought to determine whether MYCN participates in *LFG* repression. Our first approach consisted in using the SHEP MYCN-inducible Tet21N cell line, which constitutively over-expresses MYCN under the control of a tetracycline-responsive repressor element.<sup>17</sup> Doxycycline treatment of Tet21N resulted in a differentiated phenotype (Figure 6a) and reduced *MYCN* expression with a concomitant time-dependent upregulation of *LFG*, both at the mRNA and protein level (Figure 6b). Furthermore, the same result was obtained when MYCN was silenced using an inducible shRNA in an independent MYCN-amplified cell line such as IMR-32 (Supplementary Figure 4).

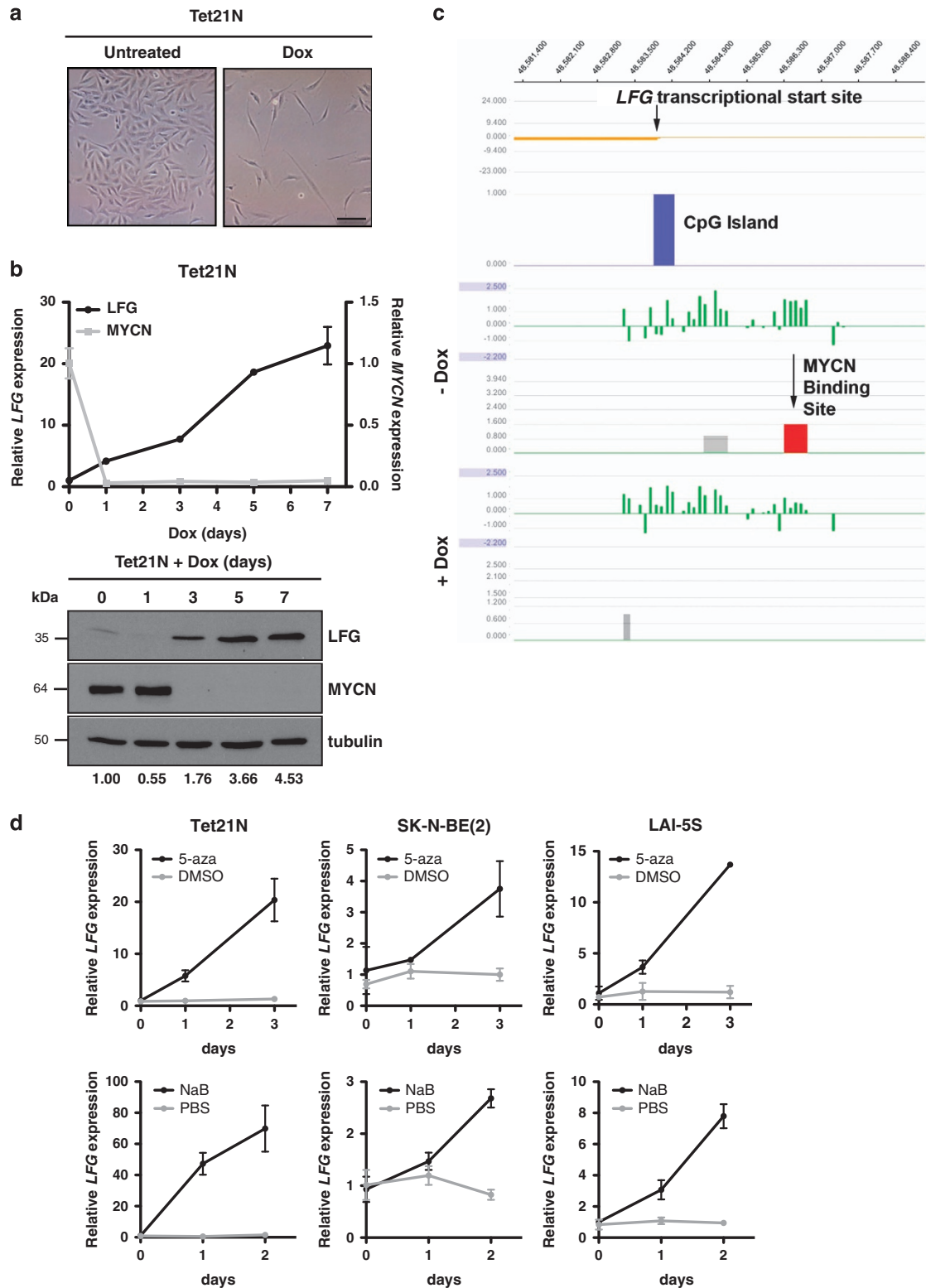


**Figure 5** LFG knockdown alters the expression of cell adhesion molecules. (a) KEGG pathway enrichment analysis of differentially expressed genes between shScr and shLFG-transduced SH-SY5Y cells. (b) Gene set enrichment analysis performed on genes differentially expressed between shScr and shLFG SH-SY5Y cells. Heatmap shows selected differentially expressed genes grouped in categories of functionally relevant pathways. (c) Array validation. qPCR using primers for three upregulated genes (*FGF14*, *STK36* and *BCL2*) and three downregulated genes (*RAP1A*, *TJP1* and *ITGA9*). *LFG* levels have been included as a control of the experiment. Values are represented as fold change versus shScr and are the mean  $\pm$  S.E.M. of three independent experiments

To address whether MYCN represses LFG directly, a chromatin immunoprecipitation (ChIP) data set was examined for MYCN binding in Tet21N cells with and without doxycycline treatment.<sup>18</sup> A high confidence MYCN-binding peak was identified in the *LFG* proximal promoter that disappeared

when *MYCN* expression was repressed by doxycycline treatment (Figure 6c).

MYCN-mediated transcription repression is now considered to be at least as important as transcription activation.<sup>19</sup> However, the repressing mechanisms remain unclear. It has



**Figure 6** MYCN represses directly *LFG* transcription. (a) Representative images of MYCN-Tet-off-inducible Tet21N cells treated with or without doxycycline (100 ng/ml). Scale bar, 100  $\mu$ m. (b) Tet21N cell line treated with doxycycline (100 ng/ml) for the indicated times. *LFG* and *MYCN* mRNA levels were detected by qPCR and normalized versus *L27* (upper panel) and also detected by western blot (lower panel). Data on the graph are presented as mean  $\pm$  S.E.M.,  $n = 3$ .  $\alpha$ -Tubulin was used as a loading control. Bands were quantified and normalized versus  $\alpha$ -tubulin. (c) Identification of MYCN binding site at the *LFG* promoter (gene ID: *FAIM2*) by ChIP in Tet21N cells. The scale on the top of the panel indicates position on chromosome 12. Green bars represent fluorescent intensity of probes around the *FAIM2* promoter, expressed as log<sub>2</sub> ratios. The red bar represents a high confidence MYCN peak, identified by the NimbleScan peak finding algorithm. (d) *LFG* levels measured by qPCR in the indicated cell lines treated with 1  $\mu$ M of the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine or 1 mM of the HDAC inhibitor sodium butyrate or for the indicated times. Mean  $\pm$  S.E.M.,  $n = 3$



been proposed that MYCN may interact with transcription factors or transcriptional complexes, inducing DNA and histone modifications. To further characterize how MYCN represses *LFG* transcription, MYCN-amplified NBL cell lines were treated with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine and the histone deacetylase (HDAC) inhibitor sodium butyrate (Figure 6d). Both treatments induced an increase in *LFG* mRNA levels in all cell lines tested, thereby suggesting that MYCN represses *LFG* expression by favoring histone deacetylation and *LFG* DNA promoter methylation.

## Discussion

Alterations of key elements of the apoptotic pathway have been shown to be determinant in the outcome of NBLs. While characterization of the mitochondrial or intrinsic pathway elements has been extensive, limited attention to the components of the extrinsic or DR pathway has been paid. The most characterized element of the extrinsic pathway is caspase-8, which is lost in ~70% of NBLs.<sup>6,20</sup> Loss of caspase-8 has been shown to prevent DR-induced apoptosis<sup>21–23</sup> and potentiate NBL metastasis.<sup>24,25</sup> However, caspase-8 can also be prometastatic if other components of the apoptotic machinery are compromised.<sup>26</sup> Whether other modulators of the intrinsic pathway can impact similarly to caspase-8 in NBL behavior remain understudied.

Here, we have analyzed the expression of several DR antagonists in multiple NBL data sets and found that *LFG* was the most differentially expressed DR antagonist in MYCN-amplified tumors. *LFG* low levels were also found to correlate with worse overall survival. *LFG* was originally reported to be expressed exclusively in the nervous system and upregulated during the development of rat sciatic nerve.<sup>27</sup> Primary functional analyses revealed that *LFG* is able to antagonize FasL but not TNF $\alpha$ -induced cell death in T cells.<sup>28</sup> We and others confirmed that *LFG* is essential to prevent Fas-induced apoptosis in neuronal cells<sup>29</sup> by clustering with Fas receptor and blocking caspase-8 activation.<sup>30</sup> Recently, an alternative *LFG* function has been proposed. Merianda and co-workers<sup>31</sup> have demonstrated that *LFG* localization in axons is sufficient to support axonal growth in dorsal root ganglionar cells, thereby indicating that *LFG* may have DR antagonist-independent functions. Alternative functions for other DR antagonists are rare but not unique. In fact, the antiapoptotic protein PEA15 has also been linked to NBL prognosis. High levels of this protein correlate with favorable clinical features. PEA15 was shown to block NBL migration through the inhibition of ERK/RSK2 signaling, suggesting a metastatic limiting role in the progression of NBL.<sup>32</sup> Similar to PEA15, loss of *LFG* potentiates cell detachment, adherence-independent growth, migration and metastasis. However, the mechanism seems to be quite different. *LFG* knockdown impacted indirectly the modulation of cell adhesion and some epithelial-to-mesenchymal (EMT) genes (Supplementary Figure 3). One example is *ITGA9*, which is found to be downregulated in sh*LFG*-infected cells. *ITGA9* has been shown to be essential for the adhesion of rhabdomyosarcoma cells.<sup>33</sup> Other prometastatic integrins were found to be

upregulated (e.g. *ITGAV*, *ITGB1*). For example, overexpression of *ITGAV* has been found to correlate with metastasis of laryngeal,<sup>34</sup> hypopharyngeal squamous cell carcinomas<sup>35</sup> and colorectal cancer.<sup>36</sup> Moreover, *ITGAV* knockdown decreased proliferation and increased apoptosis in Hep-2 carcinoma cells.<sup>35</sup> Several tight-junction (TJ) genes were also downregulated (e.g. *TJP1*). Loss of cohesion of the TJ structure can lead to invasion and ultimately to the metastasis of cancer cells.<sup>37</sup> Regarding the EMT-related genes, we have found upregulation of *TWIST1* upon *LFG* knockdown and also an inverse correlation in human samples (Supplementary Figures 3a and b). *TWIST1* is an important EMT and metastasis promoter gene,<sup>38</sup> which has been recently found to be overexpressed in MYCN-amplified NBL.<sup>39</sup> By contrast, *ERBB3* and *KRT19* genes were found to be reduced in NBL cells infected with sh*LFG* and downregulated in NBL human samples with low levels of *LFG*. *ERBB3* belongs to the epidermal growth factor receptor family and participates in the activation of proliferation<sup>40</sup> and differentiation pathways,<sup>41</sup> whereas *KRT19* is a member of the keratin family and participates in the organization of the cytoskeleton.<sup>42</sup> The expression of *KRT19* or *ERBB3* was found to be low in patients with poor prognosis.<sup>39</sup>

Other genes such as *FGF14*, *STK36* or *BCL2* may contribute in a different manner to the metastatic process. *STK36* is a serine/threonine kinase that has an important role in the activation of the sonic hedgehog pathway, reported to be essential for NBL proliferation and tumor growth.<sup>43</sup> The upregulation of *BCL2* may also contribute to inhibit apoptosis when cells grow in anchorage-independent conditions.

Furthermore, we provide evidence that low expression of *LFG* is not a mere association with the differentiation status of the cell, but it is actively repressed by the MYCN oncogene. *MYCN* encodes a protein with a basic helix-loop-helix domain known to be a transcriptional regulator of genes controlling cell cycle and proliferation, cell invasion, angiogenesis and cell survival and death pathways (reviewed in Westermark *et al.*<sup>11</sup>). *MYCN* has been shown to participate in NBL metastasis by direct or indirect repression of integrin subunits altering cell-matrix or cell-cell interactions<sup>44</sup> or repressing Caveolin-1, whose downregulation elicits anchorage-independent growth and tumor formation.<sup>45</sup> We show that repression of *LFG* impacts on the expression of several molecules that participate in cell adhesion processes, recapitulating some of the MYCN reported effects.

In summary, our results provide a new link between MYCN amplification and early steps of NBL metastasis, and the participation of *LFG* in unprecedented functions such as cell adhesion, anchorage-independent growth and migration.

## Materials and Methods

**Reagents.** Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich (Barcelona, Spain). 5-Aza-2'-deoxycytidine was purchased from Calbiochem (San Diego, CA, USA).

**Analysis of mRNA NBL data sets.** Gene expression data of 101 and 51 primary NBL tumors (GSE3960;<sup>46</sup> GSE16237<sup>47</sup>) were used to analyze the expression of DR antagonists. The expression of each individual DR antagonist

was analyzed comparing the stage 4 MYCN-amplified tumors *versus* the other stages present in the study (i.e. stages 1, 3 and 4 without MYCN amplification). Gene expression data of human samples (GSE16476<sup>48</sup> and Oberthuer lab data set from Oncogenomics database) were used to generate Kaplan–Meier survival analysis.

**Human samples.** Primary tumor tissue samples from 38 NBL patients enrolled at the Vall d'Hebron Hospital (Barcelona, Spain) were obtained immediately after surgery and snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until processing. Tumors were examined by the pathologist to confirm NBL diagnosis and the presence of at least 80% of tumor tissue sample and histopathologic classification. All patients gave written informed consent.

**Gene expression analysis by qPCR.** Total RNA was extracted from NBL cell lines and tumor samples using the RNeasy Mini Kit (Qiagen, Las Matas, Spain). Between 0.5 and  $1\ \mu\text{g}$  of total RNA was retrotranscribed using High Capacity RNA-to-cDNA Kit (Applied Biosystems, Alcobendas, Spain). Gene expression analyses were performed using the *LFG*, *TRKB*, *FGF14*, *STK36*, *BCL2*, *RAP1A*, *TJP1* and *ITGA9* Taqman probes. Unless otherwise indicated, all expression analysis were normalized *versus* 18S housekeeping gene. *MYCN* expression was performed with SYBR green (Applied Biosystems) and normalized against *L27* using the following primers: *MYCN* – forward, 5'-CCTGAGCGATTACAGATGATGA-3' and reverse, 5'-GGTGAATGTGGTGACAGCCT-3'; *L27* – forward, 5'-AGCTGTCATCGTGAAGAA-3' and reverse, 5'-CTTGGCGATCTTCTTCTTGGC-3'. Analysis was performed using the 7900HT Sequence Detection Systems 2.3 Software (Applied Biosystems). The relative fold change in expression was determined by the comparative  $2^{(-\Delta\Delta\text{CT})}$  method.<sup>49</sup>

**Western blot.** Cell homogenates were obtained in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton) and tissue homogenates were obtained in RIPA buffer (Pierce/ThermoFisher Scientific, Lafayette, CO, USA), both supplemented with  $1\times$  EDTA-free complete protease inhibitor cocktail (Roche, Sant Cugat del Vallès, Spain). Protein concentration was quantified by a modified Lowry assay (DC protein assay; Bio-Rad, Hercules, CA, USA). Proteins (20–30  $\mu\text{g}$ ) were resolved in Tris/glycine SDS/PAGE gels and transferred to PVDF membranes (Millipore, Billerica, MA, USA). After blocking with Tris-buffered saline with Tween-20 containing 5% nonfat dry milk for 1 h at room temperature, membranes were probed with anti-LFG (AnaSpec, Seraing, Belgium) or anti-FAIM2 (H-21) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) when indicated, and anti- $\alpha$ -tubulin or anti-MYCN NCM-II-100 (Abcam, Cambridge, UK) primary antibodies overnight at  $4^{\circ}\text{C}$ . Membranes were developed with SuperSignal Dura Detection Kit (Pierce/ThermoFisher Scientific) or EZ-ECL Chemiluminescence Detection Kit (Biological Industries, Kibbutz Beit-Haemek, Israel).

**Cell lines.** SH-SY5Y, IMR-32 and IMR-5 were acquired from the American Type Tissue Collection (ATCC, Barcelona, Spain). CHLA-90 cells were obtained from Children's Oncology Group Cell Line and Xenograft Repository (Lubbock, TX, USA). SK-N-BE(2) and LAI-5S cells were purchased from Public Health England Culture Collections (Salisbury, UK). Tet21N cells were a generous gift from Manfred Schwab (DKFZ, Heidelberg, Germany). IMR-32 cells transduced with the conditional MYCN shRNA were kindly provided by Dr Frank Westermann (DKFZ, Heidelberg, Germany). All cell lines obtained directly from the tissue banks were amplified and stored in liquid nitrogen. Upon resuscitation, cells were maintained in culture for no more than 2 months. SH-SY5Y, IMR-5, IMR-32 and LAI-5S cells were grown in Dulbecco's modified Eagle's medium supplemented with 15% heat-inactivated FBS, 20 U/ml penicillin and 20  $\mu\text{g}/\text{ml}$  streptomycin. SK-N-BE(2) and CHLA-90 were maintained in Iscove's modified Dulbecco's medium, supplemented with 20% heat-inactivated FBS, 1% of insulin-transferrin-selenium, 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin. Tet21N cells were grown in RPMI (Roswell Park Memorial Institute) 1640 medium (Invitrogen, Barcelona, Spain) supplemented with 10% heat-inactivated FBS, 25 mM HEPES (Invitrogen), 200  $\mu\text{g}/\text{ml}$  geneticin (G418), 0.5  $\mu\text{g}/\text{ml}$  amphotericin B, 10  $\mu\text{g}/\text{ml}$  hygromycin B, 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin. All cultures were maintained at  $37^{\circ}\text{C}$  in a saturated atmosphere of 95% air and 5%  $\text{CO}_2$ .

**RA differentiation.** Induction of differentiation of NBL cell lines was performed as described previously by Encinas *et al.*<sup>12</sup> Briefly,  $6\times 10^5$  cells were seeded in 100 mm culture dishes previously coated with 65  $\mu\text{g}/\text{ml}$  type I

collagen (BD Biosciences, San Agustín de Guadalix, Spain). *All-trans* RA was added the next day at a final concentration of 10  $\mu\text{M}$  and maintained during 1, 3 and 5 days.

**Sphere formation.** SK-N-BE(2), IMR-5 and IMR-32 cells were grown for sphere formation in serum-free neurobasal medium (Invitrogen), supplemented with B27 (Invitrogen), 2 mM L-glutamine (Invitrogen), 20 ng/ml EGF (ProSpec-Tany Technogene Ltd, Ness-Zion, Israel), 20 ng/ml FGF2 (ProSpec-Tany Technogene Ltd), 20 U/ml penicillin and 20  $\mu\text{g}/\text{ml}$  streptomycin.

**LFG knockdown.** For loss-of-function experiments, two sets of shRNA lentiviral constructs were used: shScr and shLFG were previously described in Fernandez *et al.*<sup>30</sup> and shScr2 and shLFG2 were purchased from ThermoFisher Scientific (RHS4531-EG23017). Lentiviruses were propagated using standard procedures.<sup>50,51</sup> NBL cells were seeded at  $2\times 10^6$  cells per 60 mm dish and infected with viral supernatant for 12 h. The transduction efficiency was evaluated 3 days later by scoring green fluorescent protein (GFP)-positive cells. *LFG* knockdown was assessed by qPCR and western blot.

**Cell proliferation and adhesion assays.** Infected NBL cells were seeded in 96-well plates ( $2\times 10^4$  cells/well for proliferation assays and  $2\times 10^5$  cells/well for adhesion assays) and incubated at  $37^{\circ}\text{C}$  for the indicated times. Then, cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Crystals were dissolved in 10% acetic acid and the absorbance was measured at 590 nm.

**Migration assays.** A suspension of  $2.5\times 10^5$  SH-SY5Y NBL cells was added in serum-free media to 8- $\mu\text{m}$ -pore cell culture inserts (Falcon Discovery Labware; BD Biosciences). Cells were incubated for 8 h under standard culture conditions and complete media in the lower part of the chamber. Viable cells that had migrated to the lower transwell chamber were quantified by WST-1 assay (Roche).

**Xenografts.** A total of  $5\times 10^6$  SH-SY5Y-pLuc cells infected with shScr ( $n=10$ ) or shLFG ( $n=10$ ) were injected in the flank of female NMRI nude mice in 300  $\mu\text{l}$  of phosphate-buffered saline (PBS) and Matrigel (1:1). Tumor growth was measured every 2–3 days using an electronic caliper. At the respective scheduled surgery, 150 mg/kg D-Luciferin substrate (Promega Biotech Iberica, Madrid, Spain) was injected intraperitoneally 5 min before animals were killed. The primary tumor mass was excised and weighted. Lungs were removed to detect distant metastases. Each organ was immersed in a 300  $\mu\text{g}/\text{ml}$  D-Luciferin solution in PBS and maintained in cold conditions until *ex vivo* BLI was performed using the IVIS Spectrum from the CIBER-BBN *In Vivo* Experimental Platform located at Vall d'Hebron Research Institute (VHIR).

**mRNA microarray analysis.** Expression profiling of triplicate experimental sample groups (shScr *versus* shLFG) was performed using Affymetrix microarray platform and the GeneChip Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, USA). Two hundred nanograms of total RNA was hybridized to the arrays with the GeneChip WT Terminal Labeling and Hybridization Kit (Affymetrix). Chips were processed on an Affymetrix GeneChip Fluidics Station 450 and Scanner 3000 and normalization of the raw data (CEL files) was carried out with Robust Multichip Average algorithm.<sup>52</sup> To filter and perform differential expression analyses, a moderated *t*-test ( $P<0.05$   $\alpha$ -level) and fold-change thresholding ( $>33\%$  reproducible change) were considered. The functional annotations of resulting gene lists were performed using the Gene Ontology<sup>53</sup> and the KEGG<sup>15,16</sup> databases. To build the heatmaps, array expression values were normalized to the median and log2 transformed. Values were converted to color scale using the MultipleExperimentViewer software (TM4 Software Suite, Boston, MA, USA<sup>54</sup>).

**ChIP assays.** Details of the MYCN chip experiments, along with validation studies, have been previously published by Murphy *et al.*<sup>18</sup> Sites of enrichment were identified using the normalized log2 ratios and the NimbleScan peak finding function.

**Statistical analysis.** Statistical significance was determined by unpaired *t*-test or one-way ANOVA when indicated using GraphPad (GraphPad Prism Software, La Jolla, CA, USA). \* $P<0.05$ , \*\* $P<0.01$  and \*\*\* $P<0.001$ .

## Conflict of Interest

The authors declare no conflict of interest.

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