

ORIGINAL ARTICLE

GSK3-mediated MAF phosphorylation in multiple myeloma as a potential therapeutic target

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Multiple myeloma (MM) is an incurable haematological malignancy characterised by the proliferation of mature antibody-secreting plasma B cells in the bone marrow. MM can arise from initiating translocations, of which the musculoaponeurotic fibrosarcoma (MAF) family is implicated in ~5%. MMs bearing *Maf* translocations are of poor prognosis. These translocations are associated with elevated *Maf* expression, including *c-MAF*, *MAFB* and *MAFA*, and with t(14;16) and t(14;20) translocations, involving *c-MAF* and *MAFB*, respectively. *c-MAF* is also overexpressed in MM through MEK/ERK activation, bringing the number of MMs driven by the deregulation of a *Maf* gene close to 50%. Here we demonstrate that *MAFB* and *c-MAF* are phosphorylated by the Ser/Thr kinase GSK3 in human MM cell lines. We show that LiCl-induced GSK3 inhibition targets these phosphorylations and specifically decreases proliferation and colony formation of *Maf*-expressing MM cell lines. Interestingly, bortezomib induced stabilisation of *Maf* phosphorylation, an observation that could explain, at least partially, the low efficacy of bortezomib for patients carrying *Maf* translocations. Thus, GSK3 inhibition could represent a new therapeutic approach for these patients.

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INTRODUCTION

Multiple myeloma (MM) is an incurable B-cell neoplasm of the bone marrow with a complex array of clinical manifestations.^{1–5} MM is further divided into seven disease subtypes based on molecular heterogeneity.^{6,7} Of these, one subtype corresponds to the recurrent translocations t(14;16) and t(14;20) involving different *musculoaponeurotic fibrosarcoma (Maf)* genes juxtaposed to immunoglobulin heavy chain enhancer elements, leading to their aberrant overexpression. Although still debated due to its relatively low prevalence, this group was shown to be associated with poor overall survival in MM.^{4,5,8–12}

Maf oncoproteins are basic leucine zipper transcription factors belonging to the AP-1 super family.¹³ The *Maf* family is composed of seven members that can be classified into two subfamilies, the large and small *Maf* genes. The large *Maf* members, *MAFA*, *MAFB*, *c-MAF* and *NRL*, differ from the small *Maf* members (*MAFF*, *MAFG* and *MAFK*) by the presence of a transactivation domain in their amino terminus.¹³ We and others demonstrated that large *Maf* proteins display strong transforming activity in chicken embryo fibroblasts, with *MAFA* being the most potent oncoprotein.^{14–16} We showed that *MAFA*-transforming activity in chicken embryo fibroblasts requires its phosphorylation by GSK3.¹⁷ Indeed, *MafA* is sequentially phosphorylated by GSK3 on residues S61, T57, T53 and S49.^{17,18} At the molecular level, these phosphorylations coupled two antagonistic processes. GSK3-mediated *MAFA* phosphorylation increased its transactivation activity but also induced its degradation.¹⁷

Approximately 5% of MMs bear *Maf* translocations that are associated with elevated *Maf* expression including *c-MAF*, *MAFB* and *MAFA*.^{7,19–21} The t(14;16) and t(14;20) translocations involving *c-MAF* and *MAFB*, respectively, are much more prevalent, with less

than 0.5% of MMs displaying *MAFA* translocations.^{1,4,5,7,13} In ~50% of MM patient samples lacking *Maf* translocations, *c-MAF* expression was observed at levels above those in normal plasma cells.^{22,23} *Maf* overexpression has a causative role in MM²² by regulating cyclin D2, integrin β 7, CCR1, ARK5 and DEPTOR expression, all of which have an important role in the pathogenesis of MM.^{22,24–27} These findings have been recently validated in a transgenic model, which develops B-cell lymphomas with features resembling MM-like disease highlighting *Maf* proteins as potential therapeutic targets in MM.²⁸ The latest advances in MM treatment include combination of immunomodulatory agents with the proteasome inhibitor drug, bortezomib.^{5,10,29,30} However, bortezomib appears not to be beneficial for patients carrying a *Maf* translocation.^{5,8,29} Therefore, there is an urgent need to identify additional therapeutic agents for the treatment of these patients with a poor outcome.

In the present study, we demonstrate that *MAFB* and *c-MAF*, the most frequently deregulated *Maf* in MM, are phosphorylated by GSK3, which mediates their degradation. Pharmacological inhibition of GSK3 targets these phosphorylations and leads to the decrease of MM cell proliferation and colony formation. This study provides the basis for further exploring GSK3 inhibition by lithium chloride (LiCl) in *Maf*-driven MMs in a clinical setting.

MATERIALS AND METHODS

Plasmid construction

Human *MAFB* and *c-MAF* wild type (WT) and mutated (4A) cDNAs were introduced in the pcDNA3 vector into the BamHI/EcoRI and HindIII/EcoRI restriction sites³¹ using PCR strategies.

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Cell culture and transfection

Human MM cell lines (HMCLs) RPMI8226 (RPMI), H929 (ATCC), JJN3, KMS12PE (KMS12), LP-1, L363, OPM2 (DSMZ), KMS28BM, KMS28PE and KMM1 (Japanese health sciences foundation) were cultured in RPMI1640 medium supplemented with FBS. KMS12 cells were cultured in RPMI1640–20% FBS and JJN3 in 40% DMEM, 40% IMDM and 20% FBS. For transfection, HEK293T cells were cultured in DMEM medium supplemented with 10% FBS and transfected by calcium phosphate method.

In vitro proliferation assays

Proliferation was assessed under serum-starving conditions (0.2% FBS), in the presence of the GSK3 inhibitor LiCl (Sigma, Saint-Quentin Fallavier, France). Cells were treated on day 1 with 10 mM LiCl followed by daily treatment with 2.5 mM LiCl. Cells were seeded in duplicate at a density of 2×10^5 cells per 60-mm tissue culture dish and cultured for 7 days. Live cell counts were performed on days 1, 2, 3, 5 and 7.

Half-life studies, MG132, bortezomib treatment and western blotting

Total cell extracts were prepared in TNTSE lysis buffer (140 mM NaCl, 20 mM Tris pH 8, 2 mM EDTA, NP40, 1% Triton, 0.2% SDS, 20 µg/ml aprotinin, 1 mM AEBSF, 10 mM NaF, 1 mM DTT, 25 mM β-glycerophosphate and 1 mM orthovanadate). Nuclear protein extracts were prepared according to manufacturer's guidelines (Thermo Scientific, Courtaboeuf, France). Cells were treated with 20 mM LiCl (Sigma) and the following pharmacological inhibitors: 10 µM SB216763 (Sigma), 10 µM MG132 (Sigma) and 100 nM Bortezomib (Cell Signaling Technology, Danvers, MA, USA). For bortezomib and MG132 treatment, cells were treated or not with LiCl for 1 h followed by MG132 or bortezomib treatment for 3 h. For half-life studies, cells were treated with 20 µg/ml cycloheximide (Sigma) for 30 min, 1, 2, 4 and 6 h time points in the presence or absence of LiCl.

Protein extracts were subjected to SDS-PAGE, transferred to Immobilon-P membranes (Millipore, Molsheim, France) and probed with anti-MafA (Abcam, Paris, France, 1/2000), anti-MAFB (Santa Cruz, Heidelberg, Germany, sc-10022 p20, 1/2000), anti-c-MAF (Santa Cruz, M153, 1/2000), anti-SAM68 (Santa Cruz, 1/5000), anti-β-actin (Sigma, 1/50000) and rabbit polyclonal T53-T57 phospho-specific (Rocques *et al.*,¹⁷ 1/2000) antibodies. After three washes in PBST buffer, membranes were incubated with the corresponding secondary antibody. Membranes were processed using chemiluminescence. Band intensities were measured using the G:box (Syngene, Saint Quentin en Yvelines, France) and normalised to β-actin.

Colony formation assay (Soft agar cell culture)

A mixture of 2 ml of pre-warmed (37 °C) RPMI1640 medium supplemented with 10% FBS and 2 ml pre-warmed (42 °C) 0.66% Bacto Agar Select/plate (Sigma) (0.33% final agar) was mixed with a 2×10^5 cell suspension and seeded over a 0.66% agar/full medium pre-layer (7 ml) in a 60-mm dish. The cells were allowed to grow in a humidified 37 °C incubator with 5% CO₂ for 3–4 weeks. Colony formation assays were assessed in the presence or absence of 5 mM LiCl, 10 µM GSK3 inhibitor VIII or 5 µM SB216763 (Sigma). Plates were scanned and the colony numbers were quantified using the ImageJ software.

RNA preparation and quantitative RT-PCR analysis

Total RNA extractions (Qiagen, Courtaboeuf Cedex, France) and reverse transcription (Invitrogen, Cergy Pontoise, France) were performed according to manufacturer's recommendations. Quantitative real-time PCR (qPCR) analyses were performed using the SYBR Green master mix (Life Technologies, Courtaboeuf Cedex, France). Reactions were carried out in an iCycler Thermal Cycler (Bio-Rad Laboratories, Marnes-la-Coquette, France) for 40 cycles (95 °C for 15 s/60 °C for 1 min) after an initial 10-min incubation at 95 °C. mRNA levels were normalised to levels of HPRT mRNA. All primer sequences are listed in Table 1.

Statistical analyses

Two-sided, equal variance *t*-tests were used for colony formation and qPCR assays. Data from proliferation curves between Li-treated and untreated cell lines were analysed using non-parametric Wilcoxon rank sum tests. All statistical analyses were performed using StatEL software (ad Science, Paris, France) and a *P*-value of <0.05 was considered statistically significant.

Table 1. qPCR oligonucleotides

Gene	Forward primer 5'–3'	Reverse primer 5'–3'
<i>MAFB</i>	GCTTCACCAAGGACGAGGT	GCTGCGTCTTCTCATTCTCC
<i>C-MAF</i>	GTCAGCAAGGAGGAGGTGAT	CTCCAGGACGTGTCTCTGCT
<i>DEPTOR</i>	TTTGTGGTGCAGGAAAGTAA	CATTGCTTTGTGTCACTTCTGG
<i>HPRT</i>	CCAGTCAACAGGGGACATAAA	CCTGACCAAGGAAAGCAAAG

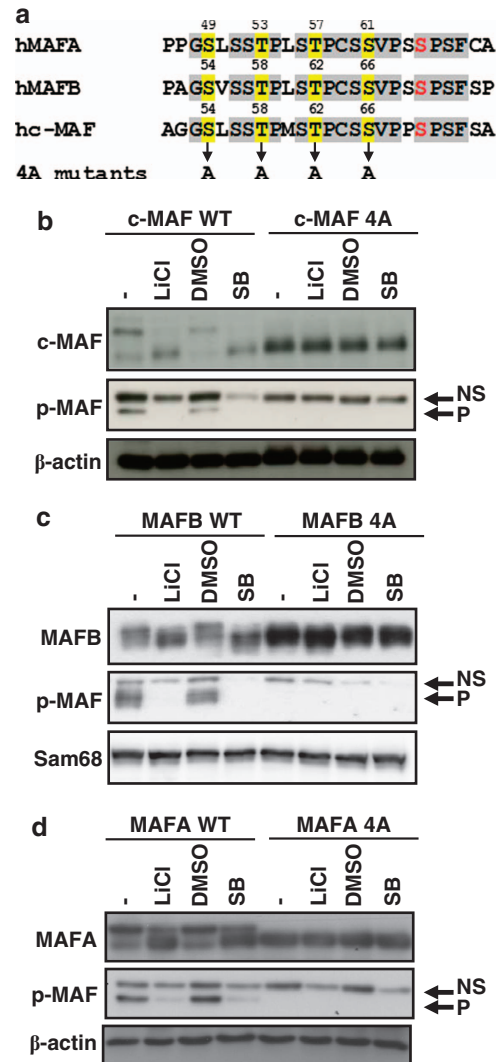


Figure 1. GSK3 phosphorylates MAFB and c-MAF proteins. (a) Amino acid sequence comparison of the large human Maf proteins (MAFA, MAFB and c-MAF). Putative GSK3 phosphorylation sites that are conserved among the different Maf proteins are highlighted in yellow. In the mutant unphosphorylatable form (4A), these putative GSK3 phosphorylation sites are mutated into alanine A. 293T cells were transfected using a pcDNA3-derived vector encoding the wild type (WT) and 4A mutants of human c-MAF (b), MAFB (c) and MAFA (d). GSK3 inhibitor treatment was done with 20 mM LiCl or 10 µM SB216763 (SB), on c-MAF, MAFB and MAFA, which demonstrates a comparable change for WT forms but had no effect on the 4A mutants. Total cell extracts were assessed by western blotting using the corresponding Maf antibodies, T53–57 phospho-specific MAFA (p-MAF) and β-actin antibodies. For MAFB (c), owing to a contaminating band in the close proximity of MAFB, nuclear extracts were used. Normalisation was carried out using an anti-Sam68 antibody. The p-MAF antibody cross-reacts with the phosphorylated forms of human c-MAF, MAFB and MAFA. NS, non-specific; P, phosphorylated forms.

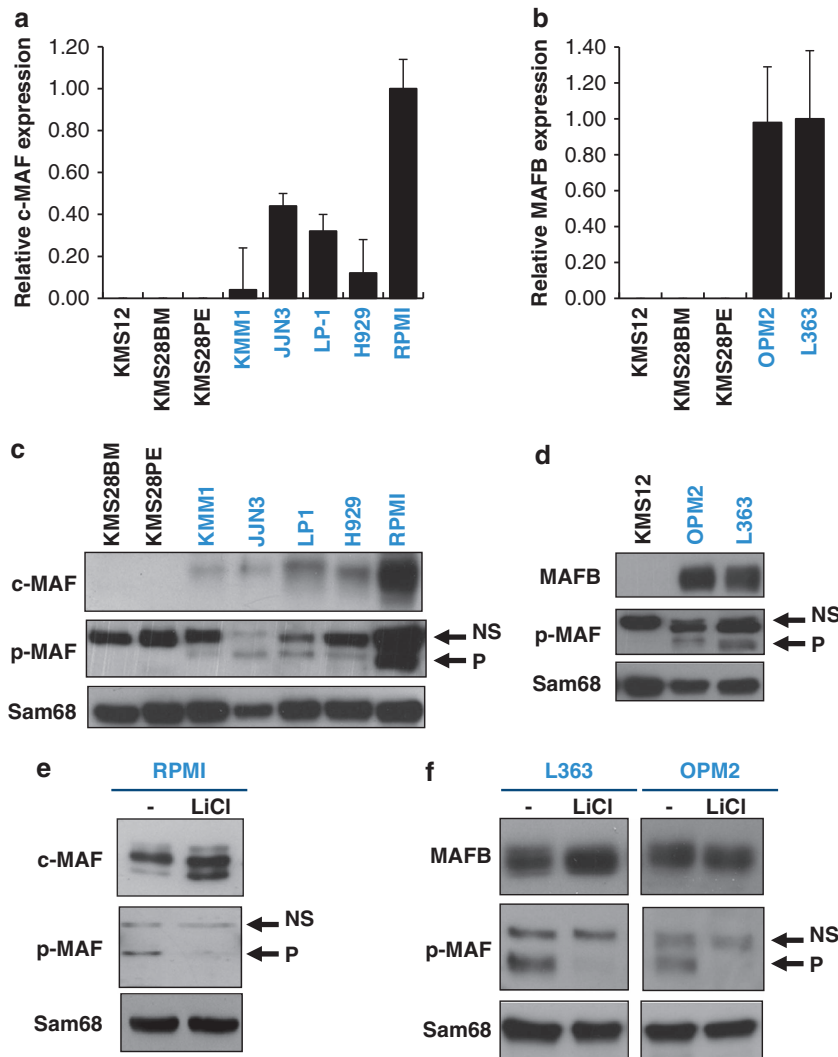


Figure 2. MAFB and c-MAF are phosphorylated by GSK3 in MM. Relative *c-MAF* (a) and *MAFB* (b) expression in MM cell lines (HMCLs) was determined by RT-qPCR. HMCLs nuclear extracts were analysed by western blot using *c-MAF* (c) and *MAFB* (d) antibodies, p-MAF and SAM68 antibodies. *c-MAF* (e) and *MAFB* (f) expressing cell lines were treated with LiCl as indicated. Anti-*c-MAF*, anti-*MAFB*, p-MAF and SAM68 western blots were carried out on nuclear extracts. NS, non-specific; P, phosphorylated forms.

RESULTS

GSK3 phosphorylates MAFB and c-MAF proteins

We previously demonstrated that MAFA is sequentially phosphorylated on residues Serine 61 (S61), Threonine 57, 53, 49 (T57, T53 and S49) by GSK3.¹⁷ These putative GSK3 phosphorylation sites are highly conserved amongst the large Maf proteins; MAFB and c-MAF (Figure 1a). In the current study, we focused on c-MAF and MAFB, the deregulation of which is the most prevalent in MM.

To investigate whether MAFB and c-MAF are phosphorylated by GSK3 on the corresponding residues, we generated constructs expressing the WT phosphorylatable forms and the mutant unphosphorylatable forms (4A) where the four putative GSK3 phosphorylation sites were mutated into alanine (Figure 1a). HEK293T cells were transfected with pcDNA3-derived vectors encoding the WT and 4A mutants of c-MAF, MAFB and MAFA as a control, and cell lysates were analysed by western blotting. Mutation of the four putative GSK3 phosphorylation sites profoundly altered the migration pattern of c-MAF (Figure 1b), MAFB (Figure 1c) and MAFA (Figure 1d). Treatment with LiCl or SB216763 (SB), two GSK3 inhibitors, led to a comparable change

for WT forms but had no effect on the 4A mutants. The migration patterns of the 4A mutants are similar to their corresponding WT forms on treatment with GSK3 inhibitors, suggesting that these residues are phosphorylated by GSK3. To confirm this phosphorylation, we made use of a previously described phospho-specific antibody that recognises T53 and T57 of chicken MAFA when dually phosphorylated (p-MAF Ab).¹⁷ This antibody also cross-reacts with the corresponding residues on MAFB (T58 and T62) and c-MAF (T58 and T62) when phosphorylated. Accordingly, the WT forms of human MAFA, MAFB and c-MAF but not the different 4A mutants were recognised by this antibody (Figure 1, middle panel). Treatment with GSK3 inhibitors prevented the detection of the WT proteins. Taken together, these results demonstrate that T58 and T62 of MAFB and c-MAF are phosphorylated by GSK3 in HEK293T. We next addressed whether c-MAF and MAFB are phosphorylated in MM.

GSK3 phosphorylates MAFB and c-MAF in MM

We undertook RT-qPCR to identify c-MAF- or MAFB-expressing HMCLs (Figure 2a). Western blot analysis was performed on

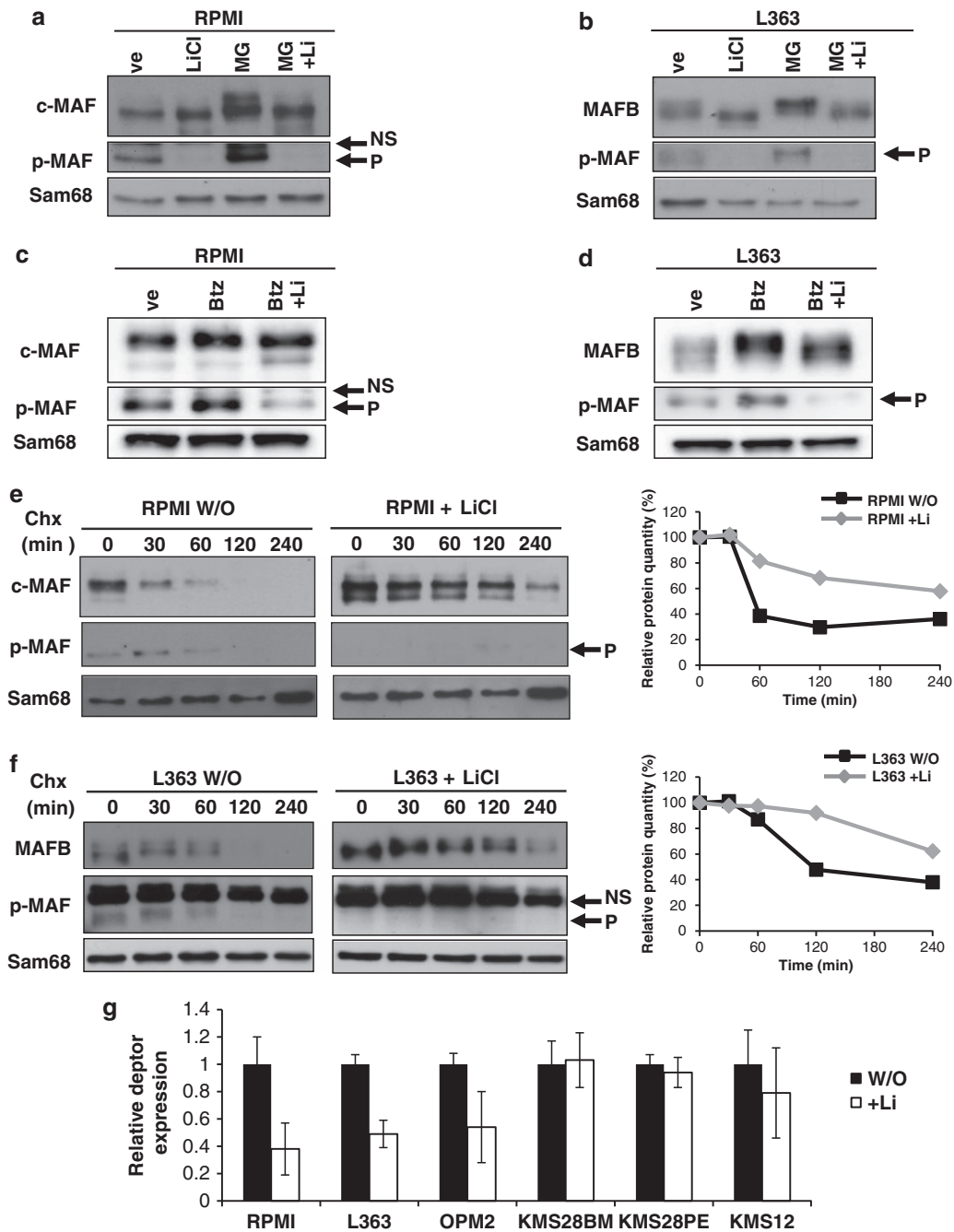


Figure 3. LiCl-mediated GSK3 inhibition leads to MAFB and c-MAF degradation and decreases *DEPTOR* expression. RPMI (a–c) and L363 (b–d) cells were treated or not with LiCl for 1 h and then with MG132 (MG) (a and b) or bortezomib (Btz) (c–d) for 3 h. Anti-c-MAF, MAFB, p-MAF and SAM68 western blots were carried out on nuclear extracts. RPMI (e) and L363 (f) cells were treated or not treated (w/o) with LiCl followed by treatment with 20 µg/ml cycloheximide (Chx) for the indicated time points. Nuclear extracts were analysed by western blots using c-MAF, MAFB, p-Maf and SAM68 antibodies. Band intensities were measured, normalised to SAM68 and plotted as a percentage of the initial band intensity (right panels). (g) The Relative *DEPTOR* expression in different HMCLs treated with LiCl (as indicated) was determined by RT-qPCR. Expression was normalised to HPRT. NS, non-specific; P, phosphorylated forms. *DEPTOR* expression was specifically decreased upon LiCl treatment in MAFB (L363 $P = 0.07$, OPM2 $P = NS$) and c-MAF-expressing cells (RPMI $P < 0.05$), but not in non-MAF-expressing cells ($P = NS$).

extracts prepared from different HMCLs, expressing MAFB (L363 and OPM2) and c-MAF (RPMI8226, H929, LP-1 and JJJ3 KMM1) and from cell lines with no endogenous expression of Maf proteins (KMS12PE (KMS12), KMS28PE and KMS28BM) as negative controls, using the p-Maf Ab (Figures 2c–f). MAFB and c-MAF phosphorylation on T58 and T62 was detected in each cell line expressing these transcription factors (Figures 2c and d). As all HMCLs express GSK3 α and GSK3 β (Supplementary Figure S2A and S2B), we assessed

whether these phosphorylations were mediated by GSK3. MM cell lines were treated with LiCl, a GSK3 inhibitor. The efficacy of this treatment was assessed through β -catenin accumulation, a known target of GSK3 (Supplementary Figure S2C). LiCl treatment led to the inhibition of MAFB and c-MAF phosphorylation (Figures 2e and f and Supplementary Figures S2D and S2E). This result demonstrates that Maf proteins are phosphorylated by GSK3 in HMCLs.

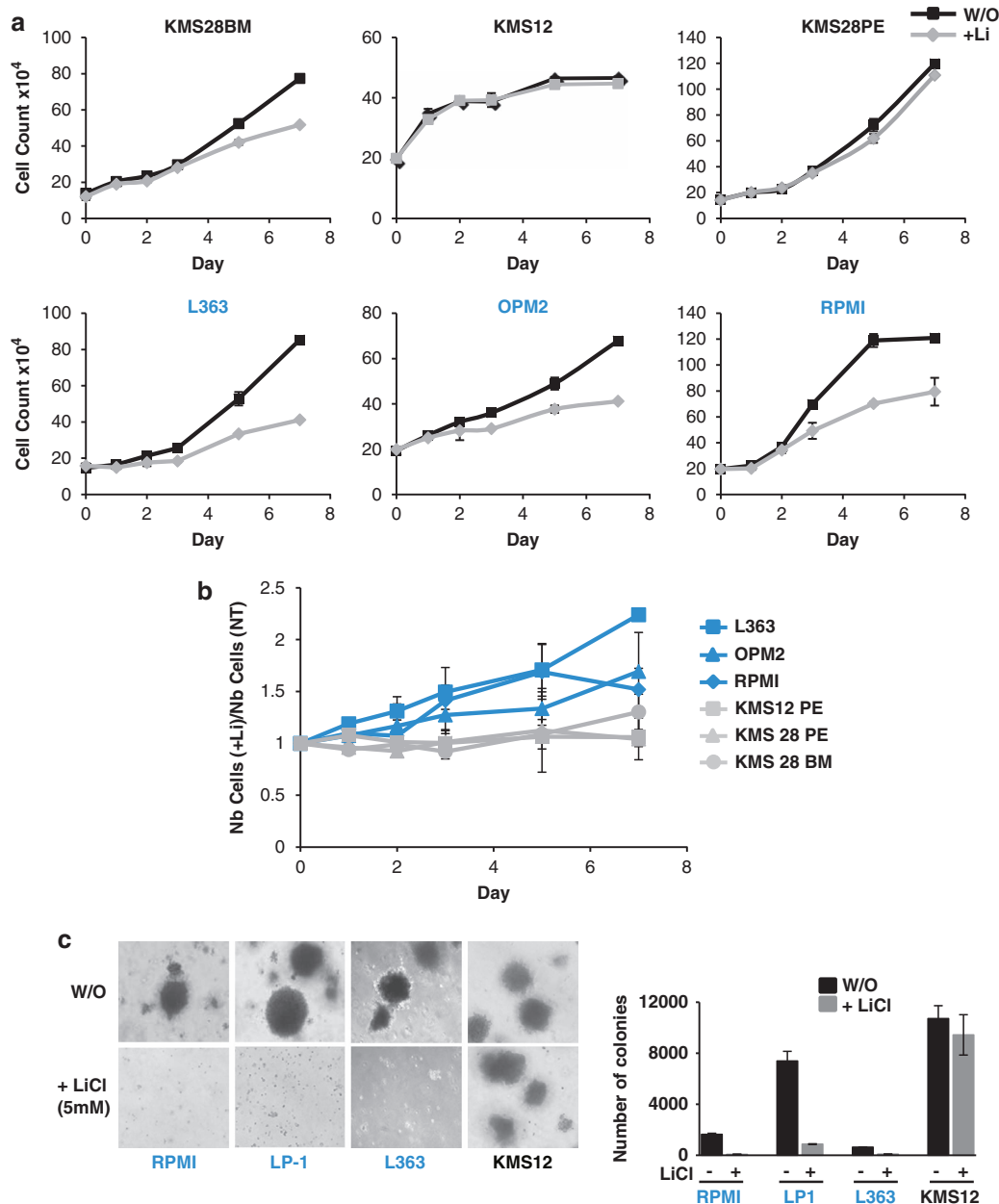


Figure 4. LiCl treatment decreases cell proliferation and colony formation in Maf-expressing MM cells. Maf-expressing cell lines (RPMI, LP-1, L363 and OPM2) are indicated in blue and non-expressing cell lines (KMS12, KMS28PE and KMS28BM) in black. (a) These cell lines were treated without (w/o) and with 10 mM LiCl. Proliferation assays were conducted and live cell counts were performed in triplicate, on days, 0, 1, 2, 3, 5 and 7. (b) To directly assess the LiCl sensitivity on the rate of HMCLs proliferation, the results obtained in a were graphed for each cell line as the number of cells in LiCl-treated culture relative to that of the non-treated condition. The average rate of proliferation of Maf-negative cell lines was significantly less affected by LiCl than c-MAF- or MAFB-expressing cell lines ($P < 0.05$). (c) Phase contrast images of soft agar assays without (w/o) and with 5 mM LiCl treatment. Plates were scanned, and the colony numbers were quantified using ImageJ and plotted for each cell line. Medium containing or not LiCl was renewed once a week for 3–4 weeks. LiCl significantly decreased colony formation in Maf-expressing cell lines; L363, LP-1 and RPMI ($P < 0.05$), it had no effect on Maf-negative cells (KMS12, $P = NS$).

GSK3-mediated phosphorylation induces MAFB and c-MAF degradation in MM

We next assessed whether these phosphorylations alter Maf stability in MM. Treatment with the proteasome inhibitors MG132 and bortezomib lead to the stabilisation of the slower migrating bands of both c-MAF and MAFB (Figures 3a–d, upper panel) that correspond to the phosphorylated forms. This result was confirmed using the p-Maf Ab (Figures 3a–d, middle panel). T58 and T62 phosphorylation of MAFB and c-MAF increased on MG132 and bortezomib treatment, whereas disappearing on LiCl

treatment, confirming that GSK3-mediated Maf phosphorylation leads to their degradation through the proteasome. To further support this conclusion, L363 (MAFB expressing) and RPMI (c-MAF expressing) cell lines were used for half-life studies (Figures 3e and f). Cycloheximide treatment demonstrated a shorter half-life for the phosphorylated forms of both c-MAF and MAFB and a much longer half-life in dephosphorylated forms induced by LiCl treatment. These results were confirmed on the WT and 4A forms of c-MAF and MAFB in 293T cells (Supplementary Figure S1). Taken together, these results show that GSK3-mediated MAFB and

c-MAF phosphorylation leads to their degradation through a proteasome-dependent pathway in MM.

The expression of the MAF target gene *DEPTOR* is sensitive to LiCl treatment

We investigated whether inhibition of GSK3 could lead to a modulation of Maf target gene expression in HMCLs. We examined the effect of GSK3 inhibition on LiCl treatment on the expression level of different Maf target genes. No changes were detected for *ITGB7* and *CCND2* expression (data not shown). In contrast, *DEPTOR* expression was specifically decreased on LiCl treatment in MAFB- and c-MAF-expressing cells, but not in non-Maf-expressing cells (Figure 3e). Therefore, phosphorylation of c-MAF and MAFB appears to be correlated with an induction of *DEPTOR* expression, although their stability is decreased.

LiCl treatment decreases cell proliferation and colony formation in Maf-expressing HMCLs

As LiCl was able to target Maf phosphorylation in MM, we next investigated whether Maf-expressing HMCLs were sensitive to this GSK3 inhibitor. We first assessed whether LiCl treatment could affect cell proliferation. For this purpose, we performed growth curve analyses under low serum conditions on different HMCLs expressing (RPMI, L363 and OPM2) or not expressing (KMS12, KMS28BM and KMS28PE) Maf proteins in the presence or absence of LiCl. As shown in Figure 4, a significant correlation was observed between Maf-expressing cells and sensitivity to LiCl. Interestingly, the rate of proliferation of Maf-negative cell lines was significantly less affected by LiCl than c-MAF- or MAFB-expressing cell lines (Figures 4a and b). To gain further insight into the effect of LiCl on Maf-mediated transformation in MM, we tested the effect of LiCl on colony formation under semi-solid conditions (Figure 4c). We seeded L363, RPMI, LP-1 and KMS12 on semi-solid medium in the presence and absence of LiCl. Although LiCl significantly decreased colony formation in Maf-expressing cell lines, L363, LP-1 and RPMI, it had no effect on Maf-negative cell lines (KMS12). These results were confirmed using two structurally unrelated GSK3 inhibitors (GSK3 inhibitor VIII and SB216763) (Supplementary Figure S2 and S3). In conclusion, Maf-expressing HMCLs display sensitivity to LiCl.

DISCUSSION

Owing to its negative effect on β -catenin stability, GSK3 is usually thought to have an inhibitory role in tumorigenic processes. However, several studies reported a positive role for GSK3 in cancer.^{32–34} For example, it represents a potential therapeutic target in mixed-lineage leukaemia.^{35,36} Recently, it was also demonstrated to have a pro-survival function in MM by permitting the activation of the non-canonical NF κ B pathway.³⁷ In this study, we demonstrate that MAFB and c-MAF are phosphorylated by GSK3 on residues T62 and T58 in MM cell lines. At the biochemical level, our results are compatible with a model where phosphorylation of c-MAF and MAFB in MM may trigger two antagonist effects, namely their destabilisation and an increase of their transactivation ability at least for a subset of target genes, as previously demonstrated for MAFA in Chicken embryo fibroblasts.¹⁷ This raises the possibility that degradation could be required for Maf transactivation, as demonstrated for other transcription factors.³⁸ We show that these phosphorylations can be targeted by the GSK3 inhibitor, lithium, a therapeutic agent already approved for patients suffering from mood disorders.^{39,40} Importantly, proliferation and colony formation of Maf-expressing cells are particularly sensitive to LiCl-mediated GSK3 inhibition. Our results suggest that the maintenance of these phosphorylations is essential for MAFB- and c-MAF-transforming activity in MM. Interestingly, proteasome

inhibition by bortezomib leads to the stabilisation of Maf phosphorylation and overall Maf accumulation, an observation that could explain, at least partially, the low efficacy of bortezomib for patients carrying *Maf* translocations. In conclusion, aside from its role in non-canonical NF κ B signalling, GSK3 also controls Maf phosphorylation, which could constitute a therapeutic target in MM. Our study suggests that patients bearing a Maf-driven MM could particularly benefit from therapies targeting GSK3.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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