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Post-Translational Regulation Contributes to the loss of LKB1 Expression through SIRT1 de-acetylase in Osteosarcomas.

Running Title: LKB1 regulation in osteosarcoma

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ABSTRACT

BACKGROUND: The most prevalent form of bone cancer is osteosarcoma (OS) which is associated with poor prognosis in case of metastases formation. Mice harbouring *LKB1*^{+/-}(Liver Kinase B1) develop osteoblastoma-like tumours. Therefore, we asked whether loss of *LKB1* gene plays a role in the pathogenesis of human osteosarcomas.

METHODS: Osteosarcomas (n=259) were screened for LKB1 and Sirtuin1 (SIRT1) protein expression using immunohistochemistry and Western Blot. Those cases were also screened for *LKB1* genetic alterations by Next Generation Sequencing, Sanger Sequencing, Restriction Fragment Length Polymorphism and Fluorescence *In Situ* Hybridization approaches. We studied LKB1 protein degradation through SIRT1 expression. MicroRNA expression investigations were also conducted to identify the microRNAs involved in the SIRT1/LKB1 pathway.

RESULTS: Forty-one percent (106 out of 259) osteosarcomas had lost LKB1 protein expression with no evident genetic anomalies. We obtained evidence that SIRT1 impairs LKB1 protein stability, and that SIRT1 depletion, leads to accumulation of LKB1 in OS cell lines resulting in growth arrest. Further investigations revealed the role of miR-204 in regulation of SIRT1 expression which impairs LKB1 stability.

CONCLUSIONS: We demonstrated the involvement of sequential regulation of miR-204/SIRT1/LKB1 in OS cases and showed a mechanism for the loss expression of LKB1 tumour suppressor in this malignancy.

Keywords: osteosarcoma, LKB1, SIRT1, post-translational regulation, miR-204, gene alteration

INTRODUCTION

Osteosarcoma (OS) is the most common non-hematopoietic, primary malignant skeletal neoplasm diagnosed in adolescents and the second leading cause of cancer-related fatalities within this age group. It is the eighth most common form of childhood cancer, representing 2.4% of all malignancies, and approximately 20% of all bone cancers (Ottaviani & Jaffe, 2009). Approximately 150 new cases of OS are diagnosed in the UK annually, and this incidence is similar to that seen in the Western world (Statistics and outlook for Bone Cancer, www.cancerresearchuk.org). Despite aggressive therapeutic management, which includes neo-adjuvant chemotherapy and surgery, 30-40% of patients die within 5 years of diagnosis (Longhi *et al*, 2006; Ottaviani & Jaffe, 2009). Several studies have confirmed the complexity and high level of heterogeneity of osteosarcoma genomes with complex karyotypes (Chen *et al*, 2014; Kovac *et al*, 2015; Lorenz *et al*, 2016). Combined genomic and transcriptomic analysis revealed extensive transcript fusion including *PMP22-ELOVL5* gene fusion, recurrent rearrangements in *RB1*, *MTAP/CDKN2A* and *MDM2* genes and also frequent *TP53* aberrations (Lorenz *et al*, 2016). Furthermore, in paediatric osteosarcomas whole-exome sequencing identified *TP53* gene alterations as well as recurrent somatic alterations in the genes *RB1*, *ATRX* and *DLG2* in a significant number of tumours (Kovac *et al*, 2015).

Germ-line mutations in *LKB1* cause Peutz-Jegher Syndrome, a rare disorder predisposing to cancer and multiple gastrointestinal hamartomatous polyps (Sanchez-Cespedes, 2007; Takeda *et al*, 2007a). Loss of heterozygosity (LOH) of *LKB1* wild-type allele has been reported in 50% of tumours (gastrointestinal tract, pancreas, cervix, ovary and breast) from patients with germ-line mutations (Boardman *et al*, 1998; Giardiello *et al*, 1987; Momcilovic & Shackelford, 2015; Ylikorkala *et al*, 1999). Loss

of *LKB1* has also been reported to induce resistance to TRAIL-mediated apoptosis in OS (Takeda *et al*, 2007a). *LKB1* loss has also been associated with a more aggressive clinical phenotype in KRAS-mutant Non-small cell lung cancer (NSCLC) patients accordingly to preclinical models (Calles *et al*, 2015). In addition, knock-down of endogenous *LKB1* gives rise to dysregulation of cell polarity and invasive phenotype of breast cancer cells (Li *et al*, 2014). Moreover, mice harbouring a heterozygous germline inactivating *LKB1* mutation develop gastrointestinal polyps, liver neoplasia, and later multifocal osteogenic and osteoblastoma-like tumours (Robinson *et al*, 2008).

LKB1 is a serine/threonine kinase, which in humans is encoded by the *LKB1/STK11* gene that regulates cell polarity and functions as a tumour suppressor (Baas *et al*, 2003; Partanen *et al*, 2012). *LKB1* is an upstream kinase of adenine monophosphate-activated protein kinase (AMPK), a necessary element in cell metabolism that is required for maintaining energy homeostasis. *LKB1* is activated allosterically by binding to the pseudokinase STRAD and the adaptor protein MO25 (Baas *et al*, 2003; Boudeau *et al*, 2003; Gaude *et al*, 2011; Hawley *et al*, 2003). The *LKB1*-STRAD-MO25 heterotrimeric complex represents the biologically active unit that is capable of phosphorylating and activating AMPK and at least 12 other kinases that belong to the AMPK-related kinase family as well as other downstream targets like mTOR, S6K, RPS6 and eIF4E (Alexander & Walker, 2011; Hardie & Alessi, 2013; Hawley *et al*, 2003; Lizcano *et al*, 2004; Patel *et al*, 2014).

It has been shown previously that SIRT1 antagonises *LKB1*-dependent AMPK activation through promoting the deacetylation, ubiquitination and proteasome-mediated degradation of *LKB1* in a senescence model of primary porcine aortic endothelial cells (Zu *et al*, 2010). SIRT1 is a NAD⁺-dependent deacetylase protein, which plays a role in a wide variety of processes including stress resistance,

metabolism, differentiation, and aging. SIRT1 binds and regulates the activity of several transcription factors including FOXO1, FOXO3, and FOXO4, HES-1 and PPAR, NF- κ B and PGC1 (Hori *et al*, 2013; Lee & Goldberg, 2013; Wang *et al*, 2011). SIRT1 has been shown to interact with and to deacetylate the p53 tumor suppressor protein (Lain *et al*, 2008; Yamakuchi *et al*, 2008).

Although osteoblastoma-like tumours were developed in LKB1 heterozygous mice, screening 113 OS patients found no LKB1 genetic alterations and in light of those findings we investigated LKB1 expression in OS cases by hypothesizing that SIRT1 deacetylase impairs LKB1 protein and provided further information into the mechanism of expression loss in those tumors.

MATERIALS and METHODS

Tumour samples

The tumour samples were obtained from the Stanmore Musculoskeletal biobank located in the histopathology department of The Royal National Orthopaedic Hospital, and the study was approved by the Cambridgeshire two Research Ethics Service (reference 09/H0308/165), the UCL Biobank for the Health and Disease ethics committee (covered by the Human Tissue Authority licence 12055; project EC17.1).

Cell culture and transfection

The human OS cell lines SaOS2, HOB, U2OS, MNNG-HOS, HOS, ZK58, KPD, HAL and OSA were obtained from EuroBoNeT (project no LSHC-CT-2006-018814 www.eurobonet.eu) and grown in Dulbecco's modified eagle medium with 10% fetal calf serum in 5% CO₂. All cell lines have been authenticated in www.ncbi.nlm.nih.gov/biosample/ and ensured that they are not misidentified. The ZK58, KPD, U2OS, OSA, HAL and OST cell lines were wildtype (WT) for *TP53* whereas the MNNG-HOS and HOS cell lines carry *TP53* mutations, and SaOS2 has a homozygote deletion for *TP53* (Ottaviano *et al*, 2010).

For transient transfections, with morpholinos or mimic microRNAs, OS cell lines were transfected in 6-well plates with Endo-Porter (Gene Tools, LLC, OR, USA), Oligofectamine or lipofectamine 2000 (Invitrogen, CA, USA). For permanent knock down transfection, the MNNG-HOS OS cell line, known to express high level of LKB1 (Takeda *et al*, 2007b), was manipulated using four hairpin clones of the Open Biosystems (GE Healthcare, Dharmacon) pGIPZ human lentiviral shRNAmir expressing LKB1-targeting shRNA (V3LHS 69003). Cell growth was monitored using

a live imaging system, IncuCyte HD (Essen BioScience, Welwyn Garden City, UK). MCF-7 and HEK293T cells which express little and high levels of LKB1 respectively at the RNA and protein level, were grown in DMEM supplemented with 10% FBS. For the knocking-in experiment a pBABE-LKB1 construction was used (Addgene, MA, USA). The empty backbones were used as a negative control. The vectors were prepared using Maxiprep kit (Qiagen, Crawley, UK) according to the manufacturer's instructions.

Immunoblotting and Immunoprecipitation

Cells were lysed with RIPA lysis buffer (50 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 10 mM CaCl₂, 0.5% NP40) for immunoblotting. Debris was removed by centrifugation and supernatant pre-cleared with protein G PLUS beads (Santa Cruz, CA, USA), and immunoprecipitated with a rabbit IgG antibody. Pre-cleared lysates were sequentially incubated overnight at 4°C (gentle agitation) with the primary antibody (4 µg(w/v) for 2 h) and protein G plus. Immunoprecipitated complexes were resolved by SDS-PAGE (8% acrylamide) for Western blot analysis. The gels were stained with anti-acetyl-lysine antibody, Chip Grade (Abcam, Cambridge, UK). The density of stained proteins was assayed on immunoblots using Image J v1.421 (NIH, USA).

Immunohistochemistry and double Immunofluorescence labelling

Immunofluorescent analysis was performed on rehydrated paraffin-embedded full tissue sections (3-4 µm). The primary antibodies included anti-SIRT1 (1:200,ab7343), anti-LKB1 (1:100,Ley37D/G6 and ab185734) (Abcam). The conjugated antibodies, anti-mouse AlexaFluor-488 and anti-rabbit AlexaFluor-568 (Molecular Probes, Eugene, OR, USA) were used as secondary antibodies. Immunohistochemistry was

performed as described previously (Amary *et al*, 2011). Other antibodies have been listed in Supplementary table 1.

Cell cycle analysis

Cell cycle analysis was performed using CyAn ADP flow cytometry (Beckman Coulter, High Wycombe, UK). SaOS2 and HOB cells were seeded in 100 mm² plates to achieve 60-70% confluency following 24 hr. Two days after transfection with morpholino antisense oligos (GENE TOOLS LLC) blocking SIRT1 RNA, the cells were harvested, centrifuged and washed with PBS. After fixation with ice-cold 70% EtOH, they were incubated in 7-AAD or anti-BrdU-FITC (BD Pharmingen, Oxford Science Park). Data were analysed by Summit v4.3 (DakoCytomation).

Agilent Human miRNA Microarray V2 and data analysis

Total RNA was extracted (miRNAeasy kit, Qiagen Ltd, Crawley, UK) and the small RNA fraction was assessed for integrity and quality using small RNA kit Lab on Chip (Agilent Technology UK Ltd, Wokingham, UK). One hundred ng of total RNA per sample was labelled and hybridized to the Agilent Human miRNA microarray V2 following the manufacturer's recommendations (Agilent technology). Microarray slides were scanned using an Agilent microarray scanner G2 505B (Agilent technology) and the images automatically analysed using Feature Extraction TM software, version 9.5.1.1 (Agilent technology). MiRNA analysis was carried out using Bioconductor packages for the R statistical programming language (Gentleman *et al*, 2004). The (gMedianSignal) background was subtracted (gBGUsed) and the arrays normalised to each other using the 'normexp' function (Ritchie *et al*, 2007) of the Limma package. The Limma package was also used for differential expression and multiple testing was controlled using the false discovery rate (*q*-value). The expression patterns were obtained by hierarchical clustering, performed by the Cluster v.3 program and

visualised by the TreeView v.1.6 software package (Eisen et al. 1998) <http://rana.lbl.gov/EisenSoftware.htm>, and their median presented in a Heatmap.

RT-qPCR

cDNA was synthesised using the superscript III first DNA kit (Invitrogen) and used as a template in SYBR green PCR Master Mix (Applied Biosystems) to assay *LKB1*, *SIRT1* and *SIRT2* mRNA expression. MiRNAs expression were investigated using TaqMan miRNA assays technology (Applied Biosystems). All reactions were performed in triplicate. The expression of the gene was quantified using either ΔC_t or $2^{(-\Delta C_t)}$ normalised to internal controls, RNU66, GAPDH or 18s (Supplementary table 2).

LKB1 Gene analysis

Twenty-one OS samples from different patients were investigated at the locus of several SNPs along *LKB1* gene. The exons 1, 2, 3, 7 and 8 (and the surrounding intron containing the SNPs) were amplified by PCR (supplementary table 3) and cleaned using the Qiaquick PCR purification kit and analysed by electrophoresis. The status of five SNPs (Supplementary table 4) was also assessed by restriction digestion (Sobottka *et al*, 2000).

Fluorescence *in situ* hybridisation (FISH) was performed using the LKB1 BAC probes generated as previously described (Fernanda Amary *et al*, 2014). DNA BAC probes were selected from the RP11 library of the Sanger Institute: RP11-81M8 for LKB1 (Spectrum-Orange) and RP11-91H11 for the telomeric control (Spectrum-Green) on chromosome 19 (BACPAC Resource centre, Oakland, CA, USA). Both probes were tested on normal cells metaphase spread to ensure that they mapped to the right chromosome. To assess the FISH analysis, 50 nuclei were counted in each case and 4

normal controls were also used to determine the cut-off values for disomy, copy number loss and gain of *LKB1*.

Statistical analysis

Growth curves statistical significance was assessed using the Mann-Whitney test using GraphPad PRISM statistic software (La Jolla, CA, USA). $p < 0.05$ was considered statistically significant. Average values were expressed as mean \pm standard deviation (SD).

RESULTS

LKB1 expression in osteosarcomas

Forty one per cent (106 of 259) of informative human osteosarcoma samples from different patients revealed loss of LKB1 expression as assessed by immunohistochemistry (Figure 1A, Supplementary Figure 1). Thirty of these tumours (random selection) were also analysed for LKB1 expression by WB and 14 revealed loss of expression (Figure 1B). The immunohistochemistry and WB data of the primary tumours correlated in 28 of 30 cases (94%). Absence of immunoreactivity in 6 of 10 cell lines (60%) correlated with low or absent detection of LKB1 on western blot (Figure 1C).

Loss of LKB1 and activation of mTOR pathway in Osteosarcomas

Using IHC we screened for the expression of key molecules in the mTOR pathway which could modulate or be modulated by LKB1. The selected molecules studied and their eventual phosphorylation sites were (TSC2 and p-TSC2 (Thr¹⁴⁶²), mTOR and p-mTOR (Ser²⁴⁴⁸), S6K and p-S6K (Thr³⁸⁹), RPS6 and p-RPS6 (Ser^{235/236})). Representative positive and negative cases by IHC and positive controls are presented in Supplementary **Error! Reference source not found.**2 and a graphical summary in Figure 1D. We found that LKB1 was not detected in 75 of 133 (56%) of the OS cases showing mTOR pathway activation, whereas only 16 of 91 (18%) LKB1-negative cases did not show pathway activation (Figure 1E). Hence, the relative risk of having the pathway activated was 12.78 times higher if LKB1 was absent (95% confidence interval). We concluded from IHC study that the mTOR pathway is activated in a vast majority of OS cases, as shown by the phosphorylation of mTOR, TSC2, S6K or RPS6 in 87% of our cohort.

***LKB1* genetic alteration**

To investigate further the loss of LKB1 protein expression, we looked for potential genetic alterations in the *LKB1* gene. FISH of the *LKB1* locus gave informative results in 90 OS cases (total 92). Twenty-eight of 90 (31%) cases were disomic, and 58 of 90 (65%) cases showed polysomy (between 3 to 7 copies of chromosome 19 (*LKB1* locus 19p13.3)). There was no evidence of copy number loss or gene deletion in the *LKB1* locus in any of the 90 cases (Figure 1D). Expression of LKB1 protein assessed by both IHC and WB alongside the FISH results showed that 23 of 32 (72%) ($p=0.002$) of the cases were disomic for LKB1 and lacked LKB1 immunoreactivity (Figure 1E). Hence, the loss of LKB1 protein expression in OS is rarely if ever explained by loss of *LKB1* gene.

We next investigated whether loss of a parental *LKB1* allele could explain the loss of LKB1 protein expression. Twenty-one OS cases, previously studied for protein expression (immunohistochemistry and WB) were analysed for the status of 12 SNPs in exons 1, 8 and introns 2,3,7,8 (supplementary Table 4). Of 20 informative cases, 8 failed to reveal any DNA variants. Of the remaining 12, parental allelic loss at the rs34928889 locus, accompanied by copy number gain of chromosome 19 was identified in 4 cases (33.3% of the 20 cases). Although this finding could explain a copy neutral loss in *LKB1* in these cases (supplementary Table 3,4), direct sequencing of exons 1, 4, 5, 6 and 8 of the 20 informative cases failed to detect any genetic alterations. In addition, no mutations were found in exons 2, 3 and 7 in the 4 cases presenting loss of a parental allele. Furthermore, Whole Genome Sequencing with the Illumina HiSeq platform (performed at Sanger Institute as part of an on-going collaboration), on 113 OS cases also showed no significant *LKB1* genetic alterations (data not shown).

LKB1 expression at transcriptional level

To test whether loss of *LKB1* mRNA level could account for the loss of protein expression, *LKB1* mRNA levels in human OS samples and cell lines were correlated with their protein levels. The RT-qPCR for *LKB1* mRNA level was performed on 11 OS cell lines and 23 OS cases. All the OS cases expressed detectable and significant *LKB1* mRNA levels compared to the average RNA level of the positive controls (Jurkat, HF1 cell lines) (Figure 1F) regardless of LKB1 protein expression levels, analysed by WB and IHC (Figure 1F).

LKB1 tumorigenicity in Osteosarcomas

We studied LKB1 role in tumorigenicity by functional approaches. OST, SaOS2 and OSA cell lines which express very low levels of the endogenous LKB1 protein were transfected with pBABE-LKB1 (LKB1-(knock in) KI) or empty vector (ET-KI) as control and then studied for Anoikis, cell survival and proliferation. Although there was no significant difference in cell cycle profile, consistent reduced growth rate and increased apoptosis in non-adherent condition was observed in LKB1-KI cells compared to ET-KI (Supplemental Figure 5). MTS assay on SaOS2 LKB1-KI cells also showed a decreased metabolism compared to the ET-KI at different cell densities, and were statistically significant at day 2 and 3 in the lowest cell density ($p=0.0099$ and $p=0.023$ respectively) and the intermediate cell density ($p=0.0034$ and $p=0.030$. resp.). Loss of LKB1 function was investigated in MNNG-HOS cells known to express LKB1 at high level at both mRNA and protein levels. Cell based assays on knock down cells revealed that LKB1-KD cells gained a growth rate compared to the pGTC-(empty vector) ET with no difference in cell cycle profile (Figure 3A).

Inverse correlation of LKB1 and SIRT1 expression in OS

We next tested whether the loss of LKB1 expression in OS could be brought about through the protein degradation machinery, and specifically through SIRT1 function. Figures 1B, C, and F show the inverse correlation of LKB1 and SIRT1 expression in OS patients and OS cell lines. Samples expressing high level of SIRT1 expressed low amount of LKB1 and inversely samples with high LKB1 expression showed a low level of SIRT1 expression $y=-1.4068x+0.3579$, $R^2 = 0.5744$ (Figure 1H). The inverse correlation was also supported by double-immunofluorescence labelling using antibodies targeting LKB1 and SIRT1 (Figure 4C, D). However, no inverse correlation was found at mRNA levels of SIRT1 and LKB1 in those tumours. Furthermore, in tumours and OS cell lines with low or high level of LKB1 protein expression, there was no significant difference in RNA levels (Figure 1I).

SIRT1 regulates LKB1 protein expression

Treatment of SaOS2 cell line with a proteasome inhibitor, PS-341 (MG-341) resulted in LKB1 protein accumulation, the degradation of which was protected by the molecule PS-341 (Figure 2A). This was reversed by treatment with Sirtinol, a SIRT1 de-acetylase inhibitor, and morpholino antisense oligonucleotides (Figure 2B, C and Supplementary table 5). Treatment of the OS cell lines SaOS2, HOB and U2OS with Sirtinol and immuno-precipitation with the anti-LKB1 antibody SIRT1 exerted a significant effect on LKB1 acetylation (Figure 2D). SIRT1/LKB1 cell localisations were studied in SaOS2 (Figure 2E) and HOB cells (data not shown) post transfection with Morpholinos and control. In addition, inactivation/suppression of SIRT1 in SaOS2 cells resulted in growth arrest in G1 phase due to SIRT1 reduction and LKB1 accumulation in those cells (Figure 2E,F, G, H).

The MNNG-HOS cells were transfected stably with Lentivirus to knock down LKB1 expression and cell growth was monitored (Figure 3A). Subsequently, the knocked

down LKB1 cells were treated with morpholinos to suppress SIRT1 expression and the levels of LKB1 and SIRT1 expression (RNA and protein) were analysed and compared to the controls (Figure 3 B, C). A significant accumulation of LKB1 protein (100%) (Figure 3B) was observed in the absence of SIRT1 expression in the MNNG-HOS cells while the LKB1 knocked down MNNG-HOS cells lost their potential to accumulate LKB1 protein.

miR-204 regulates SIRT1 expression in OS

We analysed the 22 OS cases, characterised for SIRT1 and LKB1 status, for microRNAs expression using Agilent miRNA array in a supervised manner for SIRT1 target miRNA genes acquired from miRWalk2.0 algorithms (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/>) (supplementary table 6). The expression patterns were obtained and their median presented in a heatmap (Figure 4A). miR-29c, miR-132 and miR-204, which target SIRT1, were among the microRNAs differentially and significantly expressed between OS cases (Figure 4A and supplementary table 7). However, RT-qPCR, for those miRNAs, on osteosarcoma cases revealed that 72% (14/18) of OS had relative low expression of miR-204 normalised to RNU66 level. From those samples (n=14) 11 informative cases, 67% (11 /14), showed a high level of SIRT1 mRNA expression. In addition, we found a significant correlation ($R^2=0.0383$) between the low expression of miR-204 and high SIRT1 expression (Figure 4B). This finding was in a reverse correlation with LKB1 levels (low level of LKB1) in informative cases (inset in Figure 4B, $p < 0.05$).

miR-204/SIRT1/LKB1 sequential pathway

Double immunofluorescence staining and WB on informative OS cases showed that the expression of SIRT1 and LKB1 in protein level had a reverse correlation. Two cases

were selected based on the low (S7007) and high (S7004) levels of LKB1 expression in IHC and WB (Figure 4C, D). FISH analysis confirmed trisomy at LKB1 locus in sample S7004 (Figure 4E) while there was no significant difference in the level of RNA expression between the two cases. Although in both samples, LKB1 mRNA was found to be expressed at the same level while SIRT1 mRNA in S7004 was lower than S7007, and this coincided with a higher expression level of miR-204 in S7004 compared to that is S7007 (Figure 4F).

Since miR-204 appeared to be the most relevant microRNA in this setting, compared to the data obtained from miR-132 and miR-29c experiments, we studied the effect of miR-204 in SaOS2 cell line found to express a low level of miR-204. The cells were transfected with mimics of miR-204 and subsequently cell growth and anchorage independent growth/colony formation was assessed (Figure 5A, B and C). SIRT1 and LKB1 protein expression were also analysed and showed almost 50% reduction in SIRT1 protein levels and double expression of LKB1 in the cells transfected with miR-204 compared to the scrambled miRNA on day 4 of post transfection (Figure 5D).

DISCUSSION

The role of LKB1 in cancer has recently stimulated considerable interest. Although it has been shown that the somatic inactivation of LKB1 is associated with the development of cancer in several tissues (Ji *et al*, 2007; Shorning & Clarke, 2011; Zhong *et al*, 2006), the role of LKB1 in osteosarcoma has not been studied in depth. In this study we report the absence of LKB1 expression in 41% of OS which was not due to any genetic alterations as previously shown in concordance with Lorenz (Lorenz *et al*, 2016) and COSMIC database, <http://www.sanger.ac.uk/genetics/CGP/cosmic/>. Analysing LKB1 at mRNA level and performing miRNA Array did not support the idea of deficiency at the transcriptional level, in spite of reports showing microRNAs, miR-451 and miR-155 targeting LKB1 mRNA in glioma cells and cervical cancer cells, respectively (Godlewski *et al*, 2010; Lao *et al*, 2014). The absence of LKB1 protein expression in spite of presence of mRNA implies a post-translational mechanism. SIRT1 deacetylases have important roles in many biological pathways, including cancer development (Deng, 2009; Ji *et al*, 2007; Knight & Milner, 2012; Lim, 2007; Sanchez-Cespedes *et al*, 2002; Wingo *et al*, 2009). SIRT1 can modulate cell survival by regulating the transcriptional activities of p53, NF- κ B (Yeung *et al*, 2004), FOXO proteins (Wang *et al*, 2011), and p300 (Bouras *et al*, 2005; Li & Luo, 2011). It has been shown that SIRT1 inhibition decreases Foxp3 poly-ubiquitination, thereby increasing Foxp3 protein level (van Loosdregt *et al*, 2011). Furthermore, our group recently reported that high SIRT1 protein expression was associated with metastases in Ewing sarcoma and a poor prognosis (Ban *et al*, 2014), showing the important role of this molecule in bone tumours. In addition, here we showed that loss of LKB1 could induce constitutive mTOR activation, even in the absence of detection of p-TSC2 (Thr¹⁴⁶²) and *in vitro* gain of LKB1 in OS cell lines shows a tumorigenic function.

We provide evidence via the use of small compound inhibitors and gene suppression, that LKB1 loss is through post-translational regulation. We found that LKB1 deacetylation by SIRT1 facilitates LKB1 proteasomal degradation. A mutually exclusive/reverse expression of SIRT1 and LKB1 protein shown by WB and double immunofluorescence microscopy also support this finding. Therefore, it appears that SIRT1 impairs LKB1 protein stability and that SIRT1 depletion leads to accumulation of LKB1 in OS cell lines resulting in growth arrest. In LKB1 knock down OS cells, LKB1 is restored by SIRT1 inhibitor or morpholinos, resulting in increased proliferation. These findings reveal a possible mechanism for the loss of expression of LKB1 tumour suppressor in osteosarcoma. This is in agreement with Zu *et al.*, who showed that SIRT1 antagonises LKB1-dependent AMPK activation through promoting the de-acetylation, ubiquitination and proteasome-mediated degradation of LKB1, in a senescence model in primary porcine aortic endothelial cells (Zu *et al*, 2010). Recently it has been reported that SIRT1 expression prevents adverse arterial remodelling by facilitating HECT and RLD Domain Containing E3 Ubiquitin Protein Ligase 2 (HERC2)-mediated degradation of acetylated LKB1 and acetylation at K64, LKB1 showed an enhanced interaction with SIRT1 (Bai *et al*, 2016). However, Lan *et al.*, (2008) demonstrated that overexpression of SIRT1 in 293T cells diminished acetylation of LKB1 and caused its movement from the nucleus to the cytoplasm, where LKB1 can associate with the adaptor proteins STE20-related adaptor protein and mouse embryo scaffold protein, resulting in its own activation and subsequent activation of AMPK pathway (Lan *et al*, 2008). We showed here that Immunofluorescence co-localization of LKB1 and SIRT1 on OS cell lines (SaOS2, HOB, U2OS) treated with Sirtinol or morpholinos, led to accumulation of LKB1 in cytoplasm. We showed that LKB1 was predominantly localised in cytoplasm after SIRT1 inhibition. In addition, STRAD

always co-localised with LKB1 in cytoplasm (data not shown). Consistent with our results, of reverse correlation of SIRT1 and LKB1 function, it has been shown that Metformin, a SIRT1 activator and widely used as an anti-diabetes drug, can suppress memory of hyperglycemia stress and ROS generation in the retinas of diabetic animals through the SIRT1/LKB1/AMPK/ROS pathway (Zheng *et al*, 2012).

Investigating SIRT1 upstream regulators demonstrated the post-transcriptional regulation of SIRT1 by miRNAs and RNA binding proteins. More than 16 miRNAs have been found *in silico* and experimentally to regulate both SIRT1 expression and activity (Yamakuchi, 2012). Among them are miR-29c, miR-132 and miR-204 which have been shown to contribute to tumour progression by down-regulating the expression of SIRT1 (Bae *et al*, 2014; Saunders *et al*, 2010; Strum *et al*, 2009; Zhang *et al*, 2013). Performing mimic studies on miR-204, showed a growth arrest in SaOS2 cells which coincided with SIRT1 protein reduction and an accumulation in LKB1 protein expression. This data is in agreement with Shi *et al*., showing that miR-204 inhibits proliferation and epithelial-mesenchymal transition via SIRT1 (Shi *et al*, 2015) (Zhang *et al*, 2013) in gastric cancer cells. However, miR-204 has other target genes, which also lead to cell growth arrest (Shi *et al*, 2014; Sun *et al*, 2015; Wu *et al*, 2015; Yin *et al*, 2014). All these findings suggest a sequential regulation between those molecules. Therefore, the low expression of these miRNAs may contribute to tumour development in the context of SIRT1 up-regulation and LKB1 down-regulation in a subset of OS cases.

Despite developing osteoblastic tumours in LKB1^{+/-} mice (Robinson *et al*, 2008), we could not find any genetic alterations at the *LKB1* locus in osteosarcomas, even though osteosarcomas revealed loss of LKB1 protein expression. Furthermore, we showed that SIRT1 upregulation in OS has a significant correlation with LKB1 loss, and that SIRT1

suppression revealed a restoration and accumulation of LKB1 and growth arrest. We also demonstrated a role for miR-204 in SIRT1/LKB1 pathway which may explain the loss of LKB1 expression through SIRT1 expression and its potential in arresting growth and attenuation of anchorage independent growth in OS cells. Our findings suggest involvement of sequential regulation of miR-204-SIRT1-LKB1 in OS development. Therefore, targeting this pathway may represent a useful therapeutic approach to control osteosarcomas growth in those subsets demonstrating loss of LKB1 expression.

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Statement of author contributions

AMF initiated and supervised the project; ME, conducted the experiments, analysed the data, wrote the manuscript; LD, performed genetic screening, some IHC and FISH; NP conducted genetic screening, helping with writing the manuscript; HY performed FISH analysis; RT provided clinicopathological expertise.

Titles and legends to figures:

Figure1. LKB1 expression and its genetic alterations in osteosarcomas.

(A) IHC of LKB1 in two OS with positive immunoreactivity (left) and negative immunoreactivity (right). (B) Immunoblots of endogenous LKB1 and SIRT1 protein expression in OS tumours (n=12 cases) normalised to GAPDH expression. HEK293T was used as a positive control (C) Immunoblots on OS cell lines using anti-LKB1 and SIRT1. Control cell lines HEK-293T (positive for LKB1 expression) and MCF7 and MDA-MB (negative for LKB1) were compared for LKB1 immunoreactivity in OS cell lines and patient biopsies. (D) Expression of phosphorylated and total protein for mTOR, S6K and RPS6. (E) Association between LKB1 and mTOR pathway activation in OS. Bar chart representing the LKB1 immunoreactivity compared to the pathway activation, for $p < 5 \times 10^{-15}$ with Wilcoxon signed-rank test. (F) OS TMAs FISH results for LKB1. Copy number loss of *LKB1* was defined by a ratio of *LKB1* over telomere strictly less than 1 in at least 20% of the cells and copy number gain by a ratio strictly over 1 in at least 10% of the cells. The FISH data has been summarized in a graph. (G) Bar chart represents the correlation between occurrence of chromosome 19 polysomy and the expression of LKB1 protein in OS patients. * $p < 0.005$ with the one-tailed Fisher exact test. (H) Pearson's correlation between LKB1 and SIRT1 staining intensity in OS immunoblots of 12 samples. The immunoblots were scanned and analysed by *Image J* and data were plotted to see the relationship. The expression levels were normalised to the GAPDH and compared to the HEK293T WB bands as positive control ($r = -0.086$, $p < 0.01$). (I) RT-qPCR on OS cases with no, low and high protein expression of LKB1, also on OS cell lines with no/low and high LKB1 protein expression. $2^{-(\Delta\Delta CT)}$

indicating LKB1 mRNA expression normalised to GAPDH expression, unpaired t-test between the groups, $p > 0.05$, ns=no significant.

Figure 2. LKB1 regulation via protein degradation machinery. (A) Western blot for LKB1 on SaOS2 cells exposed for eight hours with PS-341 (2 or 5 μM) or vehicle, (B) SaOS2, HOB and U2OS cells were treated (+) or not (-) with sirtinol (50 $\mu\text{g/ml}$) for 24h then lysates were immunoblotted for LKB1 and GAPDH. (C) The SaOS2 cells were transfected with morpholinos targeting SIRT1 isoforms (5, 10 μM) or both (5 μM of each) then blotted for SIRT1 and LKB1 proteins. (D) HOB, SaOS2 and U2OS cell lines were treated either with sirtinol (50 $\mu\text{g/ml}$) or vehicle control for 24h. LKB1 was immunoprecipitated (IP) from protein lysates using Protein G and the level of acetylated LKB1 were determined using anti-acetyl-lys in western blots and indicated by arrows. (E) The SaOS2 cell lines were transfected with morpholinos (iso1 (5nM)+iso2 (5nM) or scrambled and stained for LKB1 and SIRT1 for immunofluorescence microscopy (F) The SaOS2 cells either treated with sirtinol (50 $\mu\text{g/ml}$) or transfected with morpholinos (iso1 (5nM)+iso2 (5nM)), revealed a growth arrest monitored by live cell imaging system. Statistical significance was accepted at ***p value < 0.05 and *p < 0.01*. (G) Cell cycle profile for SaOS2 cells, two days post-transfection with Morpholinos, using incorporation of PI or BrdU. Representative Flow-Cytometric data showing the cell cycle distribution and growth arrest in G1 phase in SIRT1 suppressed cells. (H) The percentage of BrdU-positive cells was determined by microscopic observation. BrdU-positive and negative cells were counted at x200 magnification and at least 200 cells were counted in each slide. The graph shows the average number of cells from

each field (at least n=10) counted in three independent experiments, the bars represent the mean. **p* value < 0.05.

Figure 3. SIRT1 suppression and growth arrest modulation by LKB1. (A) Using lentivirus expressing LKB1-targeting shRNA, LKB1 was stably knocked down (KD) (about 50%) in MNNG-HOS cells. One day after selection by puromycin (10ug/ml), MNNG-HOS KD LKB1 cells were transfected with morpholinos (iso1+iso2 (5nM)) and their growth was monitored using live imaging system, (**p* < 0.05). (B, C) The MNNG-HOS KD LKB1 cells, transfected with morpholinos or control vector, were lysed two days post transfection with morpholinos, and immunoblotted using antibodies anti- LKB1, SIRT1 or GAPDH. RT-qPCR on LKB1 and SIRT1 mRNA expression was also performed on the cells treated in the same conditions.

Figure 4. miR-204/SIRT1/LKB1 regulation in OS. (A) Supervised hierarchical clustering analysis based on the predicted microRNAs targeting SIRT1 in 22 OS showed significant differentially expressed microRNAs (miR-204, miR-29c and miR-132) with a *p*<0.05 between OS cases. Expression levels are displayed in a colour spectrum that extends from light green (low expression) to bright red (high expression). (B) miR-204 RT-qPCR on OS tumours compared to SIRT1 mRNA level expression was normalised to RNU66 and GAPDH, respectively. *p* value < 0.01. $Y = -2.1389X + 5.7927$, $R^2 = 0.0383$. In those samples, level of LKB1 protein expression were compared between the informative cases (inset graph). $R^2 = 0.1481$ (regression values) (*p* < 0.05). (C) Two representative OS cases have been shown here, for low (S7007) and high (S7004) levels of LKB1 protein expression. The WB inset also confirmed the level of LKB1 expression in agreement with IHC. (D) Double IF staining for

LKB1 and SIRT1 on same two tumours. RED cells indicate LKB1 and GREEN cells indicate SIRT1 expression. Osteoclasts with a high level of LKB1 have been marked by arrows. Stromal cells expressing both LKB1 and SIRT1 resulted in a yellow colour from the merge colours (co-location). (E) Tumours were analysed by interphase FISH. S7007 was indicated as polysomic, as the presence of at least 3 copies per cell of both LKB1 and the telomere in over 10% of the cells. Tumour S7004 was indicated as disomic. (F) RT-qPCR relative expression for miR-204, SIRT1 and LKB1 mRNAs normalized to RNU66 and GAPDH respectively.

Figure 5. miR-204 regulates SIRT1/LKB1 pathway. (A, B) Cell growth and colony formation of SaOS2 cells after transfection with mimic miR-204 (100nM) in duplicate samples. ($*p < 0.05$). (C) The graph shows the average number of colonies (at least 10 cells) from each field (at least $n=5$) counted in two independent experiments, the bars represent the mean. (D) WB for SIRT1 and LKB1 on SaOS2 cells after exposure to miR-204 mimic; protein levels as indicated by densitometry.

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