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The Cks1/Cks2 axis fine-tunes Mll1 expression and is crucial for MLL-rearranged leukaemia cell viability

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The Cdc28 protein kinase subunits, Cks1 and Cks2, play dual roles in Cdk-substrate specificity and Cdk-independent protein degradation, in concert with the E3 ubiquitin ligase complexes SCF SKP2 and Apccdc20. Notable targets controlled by Cks include p27 and Cyclin A. Here, we demonstrate that Cks1 and Cks2 proteins interact with both the MllN and MllC subunits of Mll1 (Mixed-lineage leukaemia 1), and together, the Cks proteins define Mll1 levels throughout the cell cycle. Overexpression of CKS1B and CKS2 is observed in multiple human cancers, including various MLL-rearranged (MLLr) AML subtypes. To explore the importance of MLL-Fusion Protein regulation by CKS1/2, we used small molecule inhibitors (MLN4924 and C1) to modulate their protein degradation functions. These inhibitors specifically reduced the proliferation of MLLr cell lines compared to primary controls. Altogether, this study uncovers a novel regulatory pathway for MLL1, which may open a new therapeutic approach to MLLr leukaemia.

1. Introduction

The Cks proteins are small phospho-adapters required for correct CDK substrate recognition (but not kinase activity), and more precisely, for the specification of multisite phosphorylation [1,2]. Further Cks functions in transcription and protein degradation have been reported in yeast [3,4] and mammals [5–7]. Indeed, it has been shown that Cks1 and Cks2 associate with two ubiquitin E3 ligase complexes, SCFskp2 and APCcdc20, thereby regulating degradation of Cyclin A and the CDK inhibitor p27 [7,8]. Despite redundant functions between Cks1 and Cks2 [5,9–11], sequence divergence in the Skp2-binding region results in opposing roles during degradation of the Skp2 target p27. Indeed, Cks1 brings p27 to Skp2, facilitating p27 ubiquitination and degradation, while Cks2 protects p27 from Skp2 interactions and stabilises p27 [7,8,12]. Whereas both Cks1−/− and Cks2−/− mice are viable [7,13], the Cks1 and Cks2 double knockout is embryonic lethal after the morula stage, indicating that Cks1 and Cks2 are essential for embryonic development [5]. Although many Cks1−/− and Cks2−/− mouse model phenotypes are due to altered p27 regulation, loss of Cks1 in Eμ-Myc transgenic mice (a model of human Burkitt’s lymphoma) reduces cancer progression independent of p27 regulation [14], demonstrating a role for Cks1 beyond p27 regulation. In further studies of their tumorigenic potential, Cks1 and Cks2 have not only been described to be under the control of c-Myc, but also of B-RAF and Cyclin D1 oncoproteins [15]. Moreover, CKS1B and CKS2 are frequently overexpressed in various cancers [16–19], including multiple myeloma [20,21] and breast cancer [6,22,23], correlating with increased proliferation and poor prognosis.

MLL1 is a histone methyltransferase, which modulates a gene expression signature important for embryonic and haematopoietic stem cell development [24–26]. The MLL1 protein is cleaved by Taspase 1 into N-terminal (MLL N) and C-terminal (MLL C) fragments [27,28], which require phosphorylation and inter-molecular interaction for full activity [27,29]. Furthermore, bimodal degradation of MLL1 by the
SCF<sup>SKP2</sup> and APC<sup>CDC20</sup> complexes results in a cell cycle-dependent, bi-phasic expression profile [30]. The human MLL1 gene is chiefly known for its involvement in chromosomal translocations driving mixed-lineage leukemias [31]. Leukemic rearrangements fuse the N-terminal portion of MLL1 with a variety of translocation partners to produce a mature MLL-Fusion Protein (MLL-FP), which consequently omits the MLL1 C-terminal domains [32,33]. Common MLL1 translocation partners include AFF1/AF4, MLLT4/AF6, MLLT3/AF9, MLLT10/AF10, ELL, and MLLT1/ENL [34]. Mechanistically, MLL-FLPs show diminished interaction with the SCF<sup>SKP2</sup> and APC<sup>CDC20</sup> complexes, resulting in their stabilisation [30], and the building of diverse transcriptional complexes, which override the normal histone methyltransferase activity of MLL1 [28]. These alterations lead to stable expression of developmentally important target genes (e.g. Hox genes), and aberrant activation of various signalling pathways [35]. Additionally, stabilisation of wild-type (WT) MLL1 protein in MLL1 cell lines has been revealed as an important route for competing with, and suppressing the oncogenicity of, MLL-FLPs [36]. MLL-rearranged (MLLr) leukemias are currently treated by chemotherapy, but with 5% year survival rates below 50% and 20% in paediatric and adult cases respectively, there is a critical need for more effective therapies [37].

In this study, we identified Mll1 as a Cks1/Cks2 interactor and show that Mll1 stability is controlled by the Cks proteins. Collectively, our data demonstrates a role for Cks proteins in the regulation of Wnt signalling through Mll1. This previously unknown role for the CKS1/ Cks2 axis has consequences for normal Mll1 function but, unexpectedly, also for MLL-FLP leukemic activity. These findings offer a new potential therapeutic target for the treatment of poor prognosis acute leukemias.

2. Materials and methods

2.1. Cell culture and patient samples

Mouse Embryonic Fibroblasts (MEFs) were isolated from day E13.5 embryos, and cultured in DMEM supplemented with 10% Foetal Bovine Serum (FBS) and 5% penicillin/streptomycin (ThermoScientific, Loughborough, UK) as previously described [8], and were spontaneously immortalised by the 3T3 protocol. In accordance with previously reported data [8], we confirmed that Cks1<sup>−/−</sup> MEFs have a slower cell cycle, increased G1 phase population and p27 protein level when compared to wild-type (WT) control. Conversely, Cks2<sup>−/−</sup> MEFs cycle faster, with an increased S phase population, lower p27 protein level and increased γH2AX level when compared to WT control (Fig. S1).

ML-2 (DSMZ, Braunschweig, Germany; ACC15), THP-1 (DSMZ; ACC16), KOPN-8 (DSMZ; ACC552), ML-1, and RS4; 11 cell lines and peripheral blood mononuclear cells (PBMCs) [38,39] were cultured in RPMI 1640 (ThermoScientific) with 10% FBS and 5% penicillin/streptomycin.

Diagnostic peripheral blood or bone marrow cDNA samples were obtained from the MLL Munich Leukemia Laboratory. PBMCs and cord blood mononuclear cells were obtained from healthy donors and separated using Ficoll-Paque Plus as per the manufacturer's instructions (GE Healthcare, Amersham, UK). CD34<sup>+</sup> cells were isolated using the EasySep Human CD34 positive selection kit (Stem Cell Technologies, Cambridge, UK), and cultured in StemSpan SFEM II medium for expansion supplemented with hSCF (300 ng/ml), hTPO (20 ng/ml) and hFLT3L (300 ng/ml) for optimal proliferation (Peprotech, London, UK). RT-qPCRs were performed according to standard Europe Against Cancer conditions [40].

2.2. Cell cycle synchronisation

MEFs were synchronised in G1 phase by serum starvation (1% FBS), in S phase by double thymidine block (2 mM thymidine; Sigma-Aldrich, Dorset, UK), and in M phase by nocodazole block (40 ng/μl nocodazole; Merck Millipore, Watford, UK) for 12 h.

2.3. Cell transfection

All cells were transfected by nucleofection using the Amaxa nucleofector system (Lonza, Slough, UK) with either plasmid DNA (0.5-2 μg) or siRNA (0.5-1 μM). MEFs were transfected using the P4 Primary Cell Kit and program CZ-167, and MLL-translocation cell lines using the Cell Line kit L and program A-020. The FLAG-MLL1 plasmid was a kind gift from Prof. Thomas Milne. All RNA interference knockdowns were performed with two independent siRNAs, and siRNA sequences are as follows: Non-Targeting Control (Mouse) 5′-UGGUUUAACAGUCGACUA-3′, 5′-UGGUUUAACAGUGUGUGA-3′, 5′-UGGUUUAACAGUGUGUGA-3′, 5′-UGGUUUAACAGUUGUGA-3′ Dharmaco (D-00810-10-20); Mll1 (11) 5′-GCACAGUGGUCUCCAGAUU-3′ Dharmaco (J-040631-10); Mll1 (14) 5′-CUGUGUAGAACUCCAGAUU-3′ Dharmaco (J-040631-11); Non-Targeting Control (Human) 5′-UGGUUUAACAGUCGACUA-3′, 5′-UGGUUUAACAGUGUGUGA-3′, 5′-UGGUUUAACAGUGUGUGA-3′, 5′-UGGUUUAACAGUGUGUGA-3′ Dharmaco (D00810-10-20); CKS1B (A) 5′-CGACGAGGAGUGGAGAUUU-3′ [6]; CKS1B (B) 5′-ACGAAAGCUCAGAUUUGUU-3′ [6]; CKS2 (A) 5′-CUGCAAGUGUGUGUGAAGUG-3′ [18]; CKS2 (B) 5′-GUGUGUAUGUGUGACUAATT-3′ [11].

2.4. Immunofluorescent staining, ImageStream® and confocal microscopy

For ImageStream<sup>X</sup> analysis, cells were fixed in 4% paraformaldehyde, permeabilised in 0.5% Triton X-100, and incubated overnight with a pan-β-catenin antibody (Cell Signalling Technology, Hitchin, UK (CST; #8480)) followed by anti-Rabbit-FITC (ThermoScientific). Cells were resuspended in PBS containing 2 mM EDTA and 5 μg/ml DAPI. Stained cells were analysed on an ImageStream® Mark II imaging flow cytometer using the INSPIRE application (Amnis, Seattle, WA, USA). Analysis was carried out using the IDEAS software (Amnis). Cells were selected as follows: Single cells, Focused cells, Double positive (β-catenin/ DAPI). Final selected cells were analysed using the feature and mask: Similarity_Object(M01,Brightfield,Tight),β-catenin_DAPI, which calculates Similarity (logarithmic transformation of Pearson's correlation coefficient) for β-catenin and DAPI co-localisation within the cell [41] (Fig. S3). A minimum of 10,000 cells was present in the final gate (double positive). Fisher’s discriminant ratio (Rd Median) was calculated to measure nuclear translocation of β-catenin as follows: Rd = (Median sample – Median negative control)/Median absolute deviation sample + Median absolute deviation negative control. Confocal microscopy for γH2AX was carried out as previously described [8].

2.5. Real-time quantitative PCR

Total cellular RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Crawley, UK), with on-column DNAse I digestion, as per the manufacturer’s instructions. Quantitative PCR analyses were carried out using either the Quantitect SYBR Green RT-PCR kit (MEFs – Qiagen) or the TaqMan Universal PCR Master Mix kit (MLL-translocation cell lines – Thermoscientific). All SYBR Green and TaqMan assays were run on a 7900HT Real-Time PCR system (Applied Biosystems, UK). Primers are listed in Table S1.

2.6. TCF/LEF reporter assays

Both MEFs and MLL-translocation cell lines were co-transfected with 5 μg M50 Super 8x TOPFlash (#12456; Addgene) or control M51 Super 8x TOPFlash (#12457; Addgene) and 100 ng Renilla control (pRL-TK; Promega, Southampton, UK). Luciferase activity was measured using the Dual Luciferase Assay System (Promega). Relative Luciferase Activity (RLA) was calculated as Luciferase/Renilla signal.
2.7. Cell viability and apoptosis assays

Cells were seeded in triplicate in 96-well plates (1 × 10^4 cells/ml) with or without different concentrations of inhibitor (SKP2 E3 Ligase Inhibitor C1 or NAE inhibitor MLN4924; Merck Millipore). Cell viability was assessed using AlamarBlue (ThermoScientific), according to the manufacturer’s instructions, 48 h post-treatment.

For the evaluation of apoptosis, cells were stained with Annexin V-FITC and PI (Biolegend, London, UK) following manufacturer’s instructions. A minimum of 20,000 cells were assayed for each condition on a flow cytometer (BD Canto), and analysed using FlowJo software (Tree Star, Switzerland).

2.8. Cell cycle analysis

Cells were pulsed with 10 µM EdU for 60 min, and fixed in 4% paraformaldehyde. EdU incorporation was assessed with the Click-iT EdU AlexaFluor 647 kit (ThermoScientific), following manufacturer’s instructions. At least 20,000 cells were acquired using a BD Canto flow cytometer and analysed with FlowJo software.

2.9. Immunoblot analysis

Nuclear and cytoplasmic extracts were prepared using the ThermoScientific NER kit as per manufacturer’s instructions. Whole cell extracts were prepared using RIPA buffer. Western blots of MLLN and MLLC subunits were carried out on NuPAGE 3–8% Tris-Acetate gels (ThermoScientific) as per the manufacturer’s instructions.

Primary antibodies used in this study were as follows: H3 (06-755) and γH2AX (JBW301) (Merck Millipore); Actin (#3700), pan-β-catenin (#8480), Tubulin (#2128), Pin1 (#3722), phospho-AKT Ser473 (#4060) and AKT (#4691) (CST); p27 (610241) (BD Biosciences, Oxford, UK); MLL1 (A300-086A), AF9 (A300-596A), AF8 (A300-596A), AF6 (A302-199A) and ENL (A302-268A) (Bethyl, UK); MLL1C (sc-374,392), pan-Cks (sc-6238), CDK2 (sc-163), Cleaved-Caspase 3 (sc-7272) and normal IgG (Santa Cruz, CA, USA); and Cks1 (37-0200) and Cks2 (37-0300) (ThermoScientific).

2.10. Co-immunoprecipitation assay

Whole cell lysates were pre-cleared with protein G sepharose beads (GE Healthcare), and incubated overnight at 4 °C under constant rotation, with either anti-FLAG M2 affinity gel (A2220; Sigma-Aldrich) or protein G sepharose beads with the relevant antibody. 2% of each pre-cleared sample was kept as input control. Beads were washed in RIPA buffer, and protein complexes were eluted with 3X FLAG peptide (F4799; Sigma-Aldrich) or in Laemmli buffer.

2.11. Colony formation assay

Indicated cell numbers were plated in 2 ml cytokine supplemented methycellulose (Methocult H4434, Stem Cell Technologies) and incubated for 14 days before scoring for presence of phenotypic colonies according to the manufacturer’s instructions.

3. Results

3.1. Cks1 and Cks2 control Mll1 protein levels and influence Mll1-dependent gene expression

The first evidence that the Mll1 protein may be under the control of the Cks1/Cks2 axis is based on Mll1 biphasic, cell-cycle dependent regulation by the SCFSkp2 and APC^Cdc20 ubiquitin E3 ligase complexes, both of which require Cks for degradation of a portion of their known targets. Further to this, in silico analysis revealed that Mll1 contains multiple putative Cks1 minimum consensus sites ([FILP][WVY][X]TP on both N- and C-terminal subunits [2,42]. To investigate whether Mll1 protein levels, similar to p27 [8], are controlled by the Cks1/Cks2 axis, we used WT, Cks1^-/- and Cks2^-/- MEFs (Fig. S1), that were synchronised in G1, S and M phases of the cell cycle, to analyse Mll1 expression at both the RNA and protein levels (Fig. 1A-B). Mll1 transcript abundance was significantly altered in S phase for both Cks1^-/- (> 2-fold higher; p = 0.011) and Cks2^-/- (≥ 2-fold lower; p = 0.012) compared to WT. The same trend was observed in asynchronous cells, however, only the increased mRNA levels in Cks1^-/- MEFs was significantly different from control levels (≥ 1.5-fold higher; p = 0.016; Fig. 1A).

Mll1 protein levels do not directly correlate with mRNA levels, and the predominant route of Mll1 regulation has been reported to be via protein degradation [30]. To examine whether Cks proteins modulate Mll1 protein stability, we used western blotting to compare the levels of both Mll1^−/− and Mll1^−/− subunits in Cks1^-/- and Cks2^-/- MEFs. This revealed increased Mll1^−/− as well as Mll1^-/- levels in Cks1^-/- MEFs, both in asynchronous cells and at synchronised cell cycle stages. The relative increase was most striking in G1 and M phase cells and less pronounced in S phase cells. In contrast, both Mll1 subunits were almost undetectable in Cks2^-/- cells when compared to WT controls (Fig. 1B). Remarkably, this is reminiscent of the cell cycle regulator p27, whose degradation by SCFSkp2 is promoted by Cks1 and is protected from degradation by Cks2 [8].

To determine whether Cks proteins directly interact with Mll1 to influence its stability, or this effect was an indirect downstream consequence of Cdk activity changes (e.g. as seen for γH2AX [8]), we performed co-immunoprecipitation assays. WT MEFs overexpressing FLAG-tagged Cks1 or Cks2 were lysed and incubated with FLAG-conjugated agarose beads, to pull down Cks proteins. In these conditions, Cks1 and Cks2 were consistently found to pull down both Mll1^−/− and Mll1^-/- proteins (Fig. S1C). Moreover, these results strongly suggest that Cks proteins regulate Mll1 protein levels in a fashion analogous to their opposing roles in p27 degradation. Indeed, Cks1 would promote Mll1 degradation, while Cks2 would act to stabilise Mll1 by protecting it from Cks1-mediated degradation.

The histone methyltransferase function of Mll1 is critical for promoting transcription of an array of target genes, with Mll1^-/- MEFs showing significant reductions in expression of these target genes compared to WT controls [26]. Comparison of WT versus Cks1^-/- or Cks2^-/- total RNA, from asynchronous and synchronised cells, revealed major alterations in those Mll1 target genes defined by Wang et al. [26] as most frequently downregulated (e.g. Dkk3, Thbd, Hoxc9 and Mgp; Fig. 1D). Interestingly, the differences in Mll1 target genes underlined the overlapping and independent functions of Cks1 and Cks2. Whereas the changes in Dkk3, Thbd, Rspo and Mgp were similar between Cks1^-/- and Cks2^-/- cells, a subset of Mll1 target genes (e.g. Sfrp1, Tgfbi and Ebf3) was differentially regulated between Cks knockout cells. This indicates that the alteration of Mll1 protein levels in Cks-deficient MEFs has a direct impact on the primary cellular function of Mll1.

3.2. Opposing roles of Cks1 and Cks2 in Wnt signalling through Mll1 regulation

A subset of Mll1 target genes (e.g. Sfrp1, Rspo2), which are significantly altered in Cks-deficient MEFs (Fig. 1D) are key players in the Wnt signalling pathway. To study Wnt signalling in Cks-deficient MEFs, we used the TOPFlash reporter assay for β-catenin-induced T-cell factor/lymphoid-enhancing factor (TCF/LEF) transcriptional activity in response to Wnt3a, a Wnt ligand commonly used for in vitro studies [43]. This revealed a significant contribution of Cks proteins to TCF/LEF transcriptional activity (Fig. 2A). Indeed, Cks1^-/- MEFs displayed a significantly reduced basal activity compared to WT (p = 0.01), and were unresponsive to Wnt3a treatment (versus WT p < 0.05). Conversely, Cks2^-/- MEFs had a similar basal activity level compared to
which impacts on transcriptional regulation and downstream signalling (not shown). The partial restoration of Wnt signalling in Mll1−/− MEFs was observed with the FOPFlash negative control construct (data extracted from asynchronous (Async) and synchronised MEFs was analysed for the top downregulated Mll1 target genes as designated by Wang et al. [26]. Two independent control genes (Actin and Gapdh) were used to measure relative RNA abundance. Actin controlled values are represented as Log2 fold change expression versus WT control from the same cell cycle stage. Data analysis was performed using GraphPad Prism 7 software.

The cell cycle dependent regulation of Mll1 is altered in Cks-deficient MEFs, with downstream consequences on Mll1-dependent gene expression. (A) RNA levels of Mll1 in asynchronous (Async) and synchronized Cks-deficient MEFs. All cell cycle phases are represented as fold change versus WT control. A Student’s t-test was used to assess significance of differences (N = 3). (B) Western blots for nuclear extracts of asynchronous and synchronous MEFs. Histone H3 was used as a nuclear loading control. (C) Whole cell lysates from WT MEFs overexpressing FLAG-tagged Cks1 or Cks2 were immunoprecipitated using FLAG-agarose slurry. Western blots for p27 and Cdk2 were used as positive controls for co-immunoprecipitation. * indicates FLAG-tagged Cks. Western blots are representative of 3 independent experiments. (D) Heat map of Mll1 target gene expression in Cks-deficient MEFs. RNA extracted from asynchronous (Async) and synchronised MEFs was analysed for the top downregulated Mll1 target genes as designated by Wang et al. [26]. Two independent control genes (Actin and Gapdh) were used to measure relative RNA abundance. Actin controlled values are represented as Log2 fold change expression versus WT control from the same cell cycle stage. Data analysis was performed using GraphPad Prism 7 software.

The WT control, but showed significantly faster induction kinetics and reached greater maximal TCF/LEF activity in response to Wnt3a (p < 0.01; Fig. 2A). This demonstrates a complex contribution of the Cks1/Cks2 axis to Wnt signalling, potentially occurring through Mll1 protein regulation.

To investigate whether the different Mll1 levels observed in Cks-deficient MEFs were responsible for the opposing effects of Cks1 and Cks2 on Wnt signalling, the same experiment was repeated in MEFs after manipulation of Mll1 expression (Fig. S2). Overexpression of Mll1 reduced the induction kinetics and maximal response of TCF/LEF activity in response to Wnt3a in WT (p < 0.02 at 16 h and 32 h) and more strikingly in Cks2−/− cells (p < 0.01 at 16 h and 32 h; Fig. 2A + MLL1) MEFs. This is consistent with the possibility that reduced Mll1 levels in Cks2−/− MEFs contributed to increased Wnt signalling. As expected, since Cks1−/− MEFs already have a greater level of Mll1 compared to WT and Cks2−/− MEFs, Cks1−/− cells were unresponsive to Mll1 overexpression, and consequently are unable to further repress Wnt signalling activity. Conversely, siRNA-mediated knockdown of Mll1 in WT and Cks1−/− cells increased the basal TCF/LEF activity, and the kinetic of response by Wnt3a, to a small but significant and reproducible degree (Fig. 2A siRNA:Mll1). In Cks2−/− cells, Mll1 knockdown also significantly increased sensitivity to Wnt3a (Fig. 2A siRNA:Mll1). These effects were specific to Wnt signalling, as no activation was observed with the FOPFlash negative control construct (data not shown). The partial restoration of Wnt signalling in Cks1−/− MEFs by Mll1 knockdown again suggests that Mll1 is a target of Cks proteins, which impacts on transcriptional regulation and downstream signalling pathways. Thus, the respective impacts of Cks1 or Cks2 knockout were partially reversed by readjusting Mll1 protein levels.

A key step during Wnt signal transduction is the translocation of β-catenin from the cytoplasm to the nucleus [44]. To investigate this process, we used an imaging flow cytometer to measure the co-localisation of β-catenin with a nuclear dye [45] (Amnis ImageStream® Mk II; Fig. S3). As expected, nuclear β-catenin was significantly increased in WT MEFs in response to Wnt3a treatment (p < 0.05; Fig. 2B-C and Fig. S4). Nuclear translocation of β-catenin in Cks1−/− cells was significantly reduced compared to WT (p < 0.05), with lower basal nuclear β-catenin and minimal nuclear translocation in response to Wnt3a. In contrast, Cks2−/− MEFs had a significantly higher basal level of nuclear β-catenin compared to WT (p < 0.05), and were minimally responsive to Wnt3a, indicating that increased TCF/LEF activity is partially due to constitutively high nuclear β-catenin (Fig. 2B). Cell fractionation followed by western blot analysis confirmed decreased nuclear localisation of β-catenin in Cks1−/− MEFs, and proportionally higher nuclear β-catenin in Cks2−/− MEFs when compared to WT MEFs (Fig. 2D). As an additional readout for Wnt signalling, we analysed Akt phosphorylation. Wnt3a treatment caused Akt phosphorylation to be maintained at its basal level in Cks1−/−, and to be markedly upregulated in Cks2−/− MEFs when compared to WT control (Fig. 2E).

Taken together, these data demonstrate that opposing effects of Cks1 and Cks2 on Mll1 protein levels have a significant impact on cellular Wnt signalling in MEFs.

3.3. Aberrant CKS expression in MLL-rearranged leukaemias

Expression of CKS1B and CKS2 was investigated by RT-qPCR in a total of 65 patients diagnosed with AML, ALL or CML (Fig. 3A-B). CD34 enrichedcord blood mononuclear cells (CD34+ and peripheral blood mononuclear cells (PBMCs) from healthy donors were used as controls. CKS1B and CKS2 showed significantly increased expression in patient samples carrying MLL-AF9 (n = 20, CKS1B p = 0.0001, CKS2 p = 0.004), MLL-ENL (n = 8, CKS1B p = 0.0024, CKS2 p = 0.0194), MLL-AF6 (n = 6, CKS1B p = 0.0155, CKS2 p = 0.0456), MLL-AF4
(n = 5, CKS1B p = 0.0165, CKS2 p = 0.0031) and BCR-ABL (n = 20, CKS1B p = 0.0001, CKS2 p = 0.0002) fusions compared to PBMCs. CKS1B, but not CKS2, exhibited significantly higher expression in patient samples carrying the MLL-ELL fusion (n = 6, CKS1B p = 0.0128) compared to PBMCs (Fig. 3A-B). Whereas CKS2 expression in all leukaemic cells tested was similar to CD34+ cells, CKS1B was expressed at significantly higher levels in CD34+ cells than leukaemic patient samples. These data are in agreement with publicly available datasets,
including the MILE study [46], which also found significantly higher CKS1B and CKS2 expression in healthy bone marrow mononuclear cells compared to bulk MLLr leukaemic samples. Altogether, these analyses revealed a dysregulation of CKS expression in MLLr leukaemia, but also in BCR-ABL positive CML [47], consistent with putative roles for CKS1B and CKS2 in leukaemogenesis. CKS expression levels might also be linked to the relative stage of maturation in normal haematopoiesis (CD34+ versus PBMC).

3.4. Heterogeneous associations of MLL-FPs with CKS proteins

To determine whether CKS proteins interact with MLL-FPs, co-immunoprecipitation assays with antibodies specific to the MLL-FP (or an IgG isotype control as a negative control) were performed. Using cell lines carrying MLL-AF9 (THP-1 – myeloid lineage), MLL-AF6 (ML-2 – myeloid lineage) and MLL-ENL (KOPN-8 – lymphoid lineage) translocations, the fusion partners were pulled down and western blotted for endogenous CKS1 and CKS2 (Fig. S5). All fusion partner specific antibodies were able to immunoprecipitate the endogenous MLL-FPs, whereas IgG controls were not. When probing for CKS1 and CKS2, the MLL-AF9 fusion protein was found to retain interactions with both CKS1 and CKS2 (Fig. S5A). MLL-AF6 retained interaction only with CKS1 (Fig. S5B), while no interaction could be detected between MLL-ENL and either CKS1 or CKS2 (Fig. S5C). These results demonstrate that MLL interaction with CKS proteins is affected by oncogenic fusion partners in various ways, reinforcing the concept that MLL-FPs shape distinct protein complexes [28,48].

3.5. MLL fusion partners dictate the roles of CKS proteins in Wnt signalling

Active Wnt signalling is required for the development of leukaemic stem cells, disease progression, and, in some cases, loss of sensitivity to chemotherapeutic agents [49–52]. To explore CKS1 and CKS2 Wnt signalling contributions in MLL-translocation carrying cell lines, we again used the TOPFlash assay. In agreement with published data, we found the Wnt signalling pathway to be highly active in KOPN-8 cells [53] and active, albeit to a lesser extent, in THP-1 cells [54] (Fig. S6A). CKS1B knockdown significantly reduced Wnt signalling in KOPN-8 cells, but had no effect on THP-1 cells (Fig. 4A-B and Figs. S6B-C and S7). Conversely, CKS2 knockdown significantly increased Wnt signalling in THP-1 cells, but had no effect on KOPN-8 cells (Fig. 4A-B). CKS1B and CKS2 double knockdown produced an intermediate phenotype in KOPN-8 cells, but interestingly, further increased Wnt signalling in THP-1 cells (Fig. 4A-B). It should be noted that RNA interference targeting CKS1B, CKS2, or a combination of both siRNAs did not compromise the cell viability or proliferation of THP-1 or KOPN-8 cells (data not shown). Considering that CKS knockdowns were not highly efficient in THP-1 and KOPN-8 cells (Fig. S7), these data show the sensitivity to the CKS1/CKS2 axis on the regulation of MLL, and consequently Wnt signalling in MLL-translocation lines.

3.6. Inhibition of CKS-dependent protein degradation is cytotoxic for MLL-translocation lines

We next investigated the effect of small molecule inhibitors targeting the Cullin Ring Ligase (CRL) family of protein degradation machinery, of which CKS1 acts as a constituent. For this purpose, we used...
the small molecule inhibitor of the NEDD-8 activating enzyme (NAE), MLN4924 (or Pevonedistat), which has shown promise for targeting of protein degradation in cancer therapy, including diffuse large B-cell lymphoma and AML [55,39,56]. We also tested C1, a small molecule inhibitor of the SKP2-CKS1 degradation complex [57]. These inhibitors have both been shown to reduce p27 ubiquitination, with implications for cancer treatment [39,57–60]. Whereas NAE inhibitors stabilise a broad range of Cullin-RING-based ubiquitin ligase targets (e.g. pIkBα, CDT-1, p27, p21) [55,39,61], SKP2-CKS1 inhibitors exclusively suppress degradation of CKS1-independent SKP2 targets (e.g. p27), but not of CKS1-independent SKP2 targets (e.g. p21) [57,58]. MLN4924 treatment of THP-1 and KOPN-8 cells resulted in a large increase in p27 protein level, as previously described in a different MLL-AF9 cell line [39], confirming inhibition of the Cullin-containing SCF E3 ligase (Fig. 5A).

In agreement with a previous study in melanoma cells [57], treatment with the SKP2-CKS1 inhibitor C1 increased p27 protein levels in both cell lines. Interestingly, inhibitor treatments had opposite effects on MLL-fusion protein expression levels. Indeed, while KOPN-8 cells exhibited a decrease of MLL-ENL protein expression after MLN4924 or C1 treatment, MLL-AF9 protein level increased in THP-1 cells under these two conditions (Fig. 5A). CKS protein levels were also affected; there was an increase in total CKS protein in all cases, except in THP-1 cells treated with MLN4924 (Fig. 5A).

When THP-1 and KOPN-8 cells were challenged with C1 or MLN4924, both cell lines showed significant reductions in cell viability compared to cells treated with DMSO (vehicle), or CD34+/ and PBMCs from healthy donors (Fig. 5B). These results appear to contradict those obtained via RNA interference (i.e. showing no variation in cell viability), but this discrepancy is most likely explained by the incomplete knockdown, as previously stated (Fig. 57). To confirm the drug sensitivity of these cell lines when compared to healthy controls, clonogenic assays were performed. Colony formation of MLLr AML lines was reduced following C1 or MLN4924 treatment, compared to the vehicle control, whereas CD34+ colony formation remained unaffected (Fig. S8). The reduction in THP-1 cell viability correlated with a significant increase in apoptotic cells (Annexin V positive; Fig. 5C) and activation of Caspase 3 (Fig. S9) for both C1 and MLN4924. Surprisingly, while the KOPN-8 cells showed increased apoptosis after C1 treatment, MLN4924 caused cell cycle-arrest in G1 or G2 phase, devoid of cells progressing through S-phase (Fig. 5D and Fig. S9). We tested two additional cell lines: ML-1, an AML cell line expressing MLL-AF6, and RS4;11, an ALL cell line expressing MLL-AF4. Interestingly, both responded to the inhibitor C1, whereas MLN4924 treatment induced cell death only in the ML-1 cell line (Fig. S10). Thus, effective targeting of the neddylation pathway using MLN4924 seems to be restricted to MLLr AML lines, while both MLr and MLLr ALL lines appear to be sensitive to C1 at doses which exhibit low toxicity in healthy cells.

Altogether, these results indicate that Cullin-based protein degradation is important for MLL-translocation cell viability, and a range of SKP2-CKS1 targets, the MLL-FPs themselves included, may be involved in fine-tuning this process.

### 4. Discussion

Previous studies, describing the requirement of Cks1 [8,47] and Cks2 [8,13] in cell cycle dynamics, have postulated that targets of the Csk1/Csk2 axis may extend beyond the specific subset of Cyclins and Cdkks [5,7,10,13,62]. Here, we provide the first evidence for an essential role of the Csk1/Csk2 axis in the regulation of Mll1 expression. The divergent roles Cks1 and Cks2 play in Mll1 stability have major downstream consequences on the Wnt signalling pathway. We also identified an impact on MLLr cell viability when interfering with the Csk1/Csk2 axis.

We demonstrated the requirement of Cks1 and Cks2 in Mll1 protein expression throughout the cell cycle. Interestingly, knockout of Cks1 mimics the stable Mll1 protein levels previously described in leukaemia [30], while knockout of Cks2 has the opposite effect, with Mll1 protein levels almost undetectable. The downstream effect of Mll1 stabilisation (as observed in Csk1−/− MEFs) has been well documented in MLLr leukaemia [28,30,31,63,64], in which the normal epigenetic profile and cooperating signalling pathways, governed by MLLr, are hijacked by MLL-FPs [48,65–69]. One key downstream pathway is the Wnt/β-catenin pathway, which is involved in both primary mixed-lineage leukaemia development [45,49,50] and drug resistance [70,71]. Whilst the bone marrow niche is the primary provider of extracellular Wnt signals required for both haematopoietic stem cell and leukaemic stem cell self-renewal [51,72–74], MLL1 and MLL-FPs play critical roles in intracellular regulation of Wnt signalling [52,75–77]. Therefore, the
Fig. 5. MLL-translocation cell lines are sensitive to inhibition of pan-Cullin-dependent and CKS1-dependent protein degradation. (A) Western blots for THP-1 (MLL-AF9) and KOPN-8 (MLL-ENL) cells treated with vehicle control (DMSO), 0.1 μM MLN4924 or 1 μM C1 for 16 h and 24 h. Histone H3 was used as a loading control. Western blots are representative of 3 independent experiments. (B) Cell viability was assessed for THP-1, KOPN-8, healthy PBMC and CD34+ control cells after 3 days treatment with indicated concentrations of the pan-Cullin inhibitor (MLN4924) or the SKP2-CKS1 inhibitor (C1). Results represent the mean of 3 independent experiments with standard deviation bars. (C) Proportion of apoptotic CD34+, de

Mill1-dependent impact of the Cks1/Cks2 axis on Wnt signalling is of particular interest. The Wnt signalling changes that we observed in Cks-deficient MEFs are consistent with those reported in both the Skp2−/− [74] and p27−/− [78–80] mouse models, indicating that Cks1/Cks2/SCAP2p2 dynamics are likely responsible for these alterations. Interestingly, Cks1B (Myc-dependent) [14] and Cks2 [81] expression can also be regulated by the Wnt signalling effector β-catenin, forming a feedback loop mediated through the Cks1/Cks2 axis, which may be required to balance intracellular Wnt signalling, in response to both internal and external stimuli. In cases in which Wnt signalling is responsible for the emergence of leukaemic stem cells that are resistant to key therapeutic agents [49,70,71], the regulation of Mill1, and consequently Wnt signalling, by the Cks1/Cks2 axis provides a positive new alternative for combating these leukaemic stem cells and keeping Wnt signalling under control.

Overexpression of Cks1B and Cks2 has been documented in a variety of cancers [6,16,18–23,82], downstream of a variety of oncoproteins, and their high expression has been correlated with poor prognosis. Similarly, Cks1B expression has been reported to be high in CML patients at blast crisis and, further to this, significantly reduced after imatinib (STI-571) treatment [47]. Investigation of Cks1 roles in the Eμ-Myc lymphoma mouse model indicates that Cks1 functions in cancer expand beyond p27 regulation, and other pathways may be regulated by Cks1 [14]. We found that MLLr leukaemic blasts have upregulation of Cks1B and Cks2 compared to terminally differentiated cells (PBMCs), and further increase Cks1B expression in CD34+ cells, a finding further confirmed in the MILE study [46]. This suggests that Cks-upregulation could be a significant factor in these cancer cells. Higher expression of Cks1B and Cks2 in leukaemic cells, compared to terminally differentiated cells, may be an important part of the epigenetic re-programming of normal blood cells to block differentiation and increase the number of more stem-like leukaemic cells by MLL-FPs [28,64,83–85]. Furthermore, high levels of Cks1B observed in haematopoietic stem and progenitor cells (CD34+) indicate that Cks1B may be a central factor of the stem cell program hijacked by MLL-FPs in MLLr leukaemia.

The variety of leukaemogenic MLL-FPs reported produce a heterogeneous disease, dependent on the FP present [28,86]. This heterogeneity was reflected in the presence or absence of interactions with Cks1 and/or Cks2, depending on the respective MLL-FPs that we investigated. Despite a lack of interaction between Cks1/2 and MLL-ENL, Wnt signalling was still affected in MLL-ENL cells following Cks1 and/or Cks2 knockdown. A probable explanation is that Cks1/2 are displaced in the larger COMPASS-like complex [28] built by MLL-ENL, which might impact on additional downstream functions. Although Cks1/2 was shown to interact with Mill1 (N- and C-terminal subunits), interaction between Cks1/2 and the fusion partner or the wild-type copy of the fusion partner cannot be excluded. Further to this, stabilization of the WT Mill1 protein in MLL-FP cells may play a crucial role in combating aberrant MLL-FP epigenetic functions [36]. Disrupting
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Supplementary data

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References


