Structured Reports for the Pathological Society Grant Awards: Career Development Fellowships (CDF), Path-Soc / J Shanks Fellowship, Cuthbert Dukes Award, PhD Studentships, International Collaborator Awards, Small Grant Scheme (several types) Awards, CRUK/Path-Soc Pre-Doctoral Research Bursary & Early Career Pathology Researcher (Hodgkin & Leishman) Grants.

Recipients of grant awards from the Pathological Society of Great Britain and Ireland should submit a scientific report detailing the work undertaken with support from this award and any outputs arising from this. The reports should be set out using the following subheadings and should consist of:

Final reports: 2 A4 pages of text

Title: Splenic marginal zone lymphoma: KLF2 mutation and its molecular oncogenic mechanism

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Background and aims:

Splenic marginal zone lymphoma (SMZL) is a low-grade B cell neoplasm, which originates from the marginal zone B cells. Our previous study by whole exome sequencing identified frequent inactivating mutations in KLF2, a transcription factor that is implicated in a variety of cellular processes. We also showed that wild type KLF2 suppresses NF-κB activation *in vitro*, while *KLF2* mutations abolish its ability to suppress NF-κB activation by TLR, BCR and BAFFR signalling, which are critical for the development of marginal zone B-cells.

The proposed investigations aim:
1) to investigate whether KLF2 also regulates the NOTCH signalling that is important for marginal zone B-cell biology;
2) to identify the genes regulated by KLF2 by an integrated ChIP-Seq and RNA-Seq analysis;
3) to identify the transcriptional regulators such as transcriptional repressors and activators modulated by KLF2, and dissect its potential connection to other transcriptional factors, particularly those, such as NF-κB and NOTCH2, critical for marginal zone B-cell development.

Results:

Different effects on intracellular signalling among KFL2 wild type and mutants

To test whether KLF2 also regulates NOTCH2 activation, we performed reporter assays in HEK293T and DG75 cells. In both cell lines, the wild type KLF2 repressed CSL activities triggered by N2ICD, and all KLF2 mutants with the exception of P70S and A291V showed a total loss of this repression, displaying similar or higher CSL activities than the vector control (Figure 1B). This was particularly evident for the KLF2 C274Y, C279Y, H292Y, H296Y and S287P mutants.

To further investigate whether KLF2 may act as a broad transcriptional regulator beyond the signalling pathways important for marginal zone B-cell differentiation, we extended the reporter assays to include SRE (serum response element, typically triggered by MAPK/ERK signalling) using CCR6 stable expression cell lines (both wild type and a truncation mutant) under CCL20 simulation. Similar to the observations above, the wild type KLF2 repressed SRE activities triggered by CCL20 mediated CCR6 activation, and all KLF2 mutants with the exception of P70S and A291V showed a total loss of this repression, rather exhibiting reporter activities compatible to or higher than the vector control (Figure 1C). This was particularly evident for C274Y

and C279Y, followed by the H292Y and H296Y mutant. Of note, all these 4 mutants involve an amino acids change to a tyrosine within the ZF1 domain.

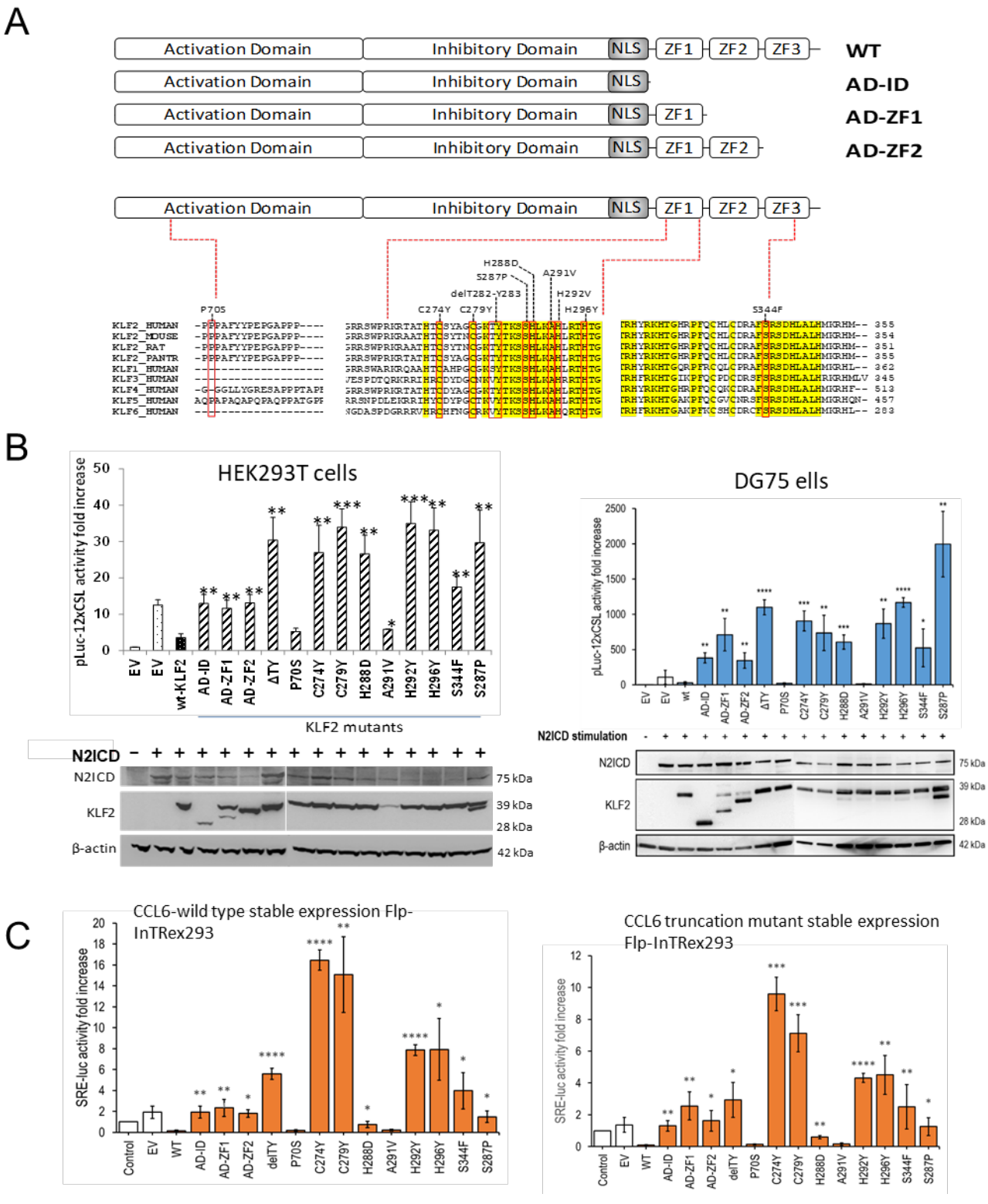

Figure 1: Different functional effects between KFL2 truncation and missense mutants
The data is from at least three independent experiments and presented as a mean ± standard deviation, and the difference between KLF2 and its mutants is analysed by the unpaired student t-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Different subcellular localisation among KFL2 wild type and mutants

In both HEK293T and DG75 cells, wild type KLF2 was exclusively expressed in the nucleus in a diffuse pattern, and largely co-localised with N2ICD (Figure 2). In contrast, the truncation mutants showed more cytoplasmic

expression, and with the extent of its cytoplasmic localisation correlating to the proportion of C-terminal deletion (Figure 2). In line with this, wild type KLF2 primarily co-localized with N2ICD, while the truncation mutants, particularly AD-ID and AD-ZF1, and were not associated with N2ICD due to their cytoplasmic displacement.

All the missense mutants were exclusively expressed in the nucleus with the ΔTY, P70S, H288D, A291V and S344F mutants showing largely a diffuse pattern similar to the wild type. While the remaining missense mutants, particularly C274Y, C279Y, H292Y and H296Y showed a punctate, speckled pattern in a high proportion of transfected cells, similar in both HEK293 and DG75 cells (Figure 2). Such nuclear speckle pattern was not seen for N2ICD and RBPJ, at least not prominently, thus most likely acquired by these KLF2 missense mutants.

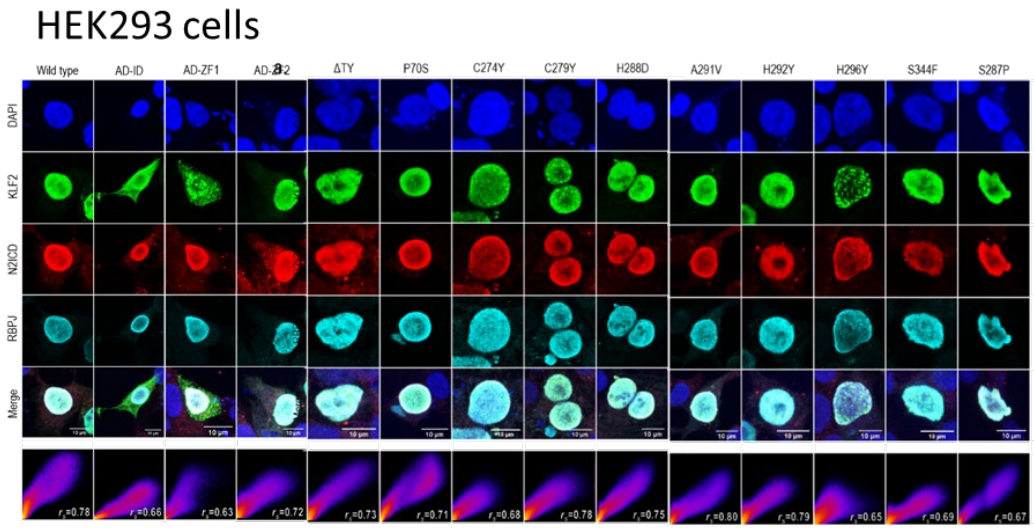

Figure 2. Different subcellular localisation between KFL2 truncation and missense mutants

As nuclear speckle expression pattern is a characteristic feature of proteins involved in spliceosome, such as serine/arginine-rich splicing factor 2 (SRSF2, also known as SC35), we next investigated whether the above KLF2 missense mutant may co-localize with SRSF2 by confocal microscopy. The KLF2 H296Y mutant did not appear to co-localise with SRSF2, but their speckles were often directly adjacent each other (Figure 3).

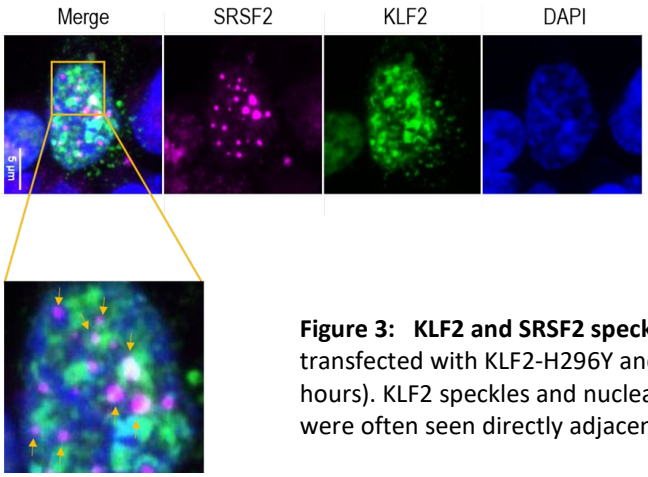

Figure 3: KLF2 and SRSF2 speckles in HEK293T cells. HEK293T cells transfected with KLF2-H296Y and treated with actinomycin D (1μg/ml for 3 hours). KLF2 speckles and nuclear speckles do not appear to overlap but were often seen directly adjacent to each other.

Evidence of interaction between KLF2 and spliceosome

To further understand KLF2 function, we investigated its interacting proteins by immunoprecipitation of nuclear protein extract, followed by SILAC mass spectrometry. Three independent experiments were performed for both KLF2 wild type and C274Y mutant. Among the 68 proteins identified, majority were involved in spliceosome regulation, including spliceosome components and splicing factors.

To further validate these findings, we performed immunoprecipitation for ADAR (double-stranded RNA-specific adenosine deaminate), a RNA editing enzyme that directly regulates the splicing process and confirmed its interaction with KLF2 wild type, C274Y and H296Y, but not the AD-ID truncation mutant. Taken together, these findings suggest that KLF2 may involve, at least in part, spliceosome regulation.

KLF2 biology through gene expression profiling analysis

To understand the biological function of KLF2 and its dysregulation by mutation, we compared the gene expression profile among DG75 cells with inducible expression of KLF2-WT, AD-ID, C274Y and H296Y. Both principal component analysis and hierarchical clustering showed three distinct clusters. All the parental cells with or without doxycycline treatment, KLF2-WT and mutant cells treated with vehicle, and KLF2-AD-ID cells treated with doxycycline at both 24 and 48 hours formed one tight cluster, while KLF2-WT cells under doxycycline induction at both 24 and 48 hours formed a separate cluster. The KLF2-C274Y and KLF2-H296Y mutant cells under doxycycline induction clustered separately, intermingled together, although those induced for different times (24 vs 48 hours) slightly separating from each other. These distinct clusters were also seen by unsupervised clustering analysis based on highly and differentially expressed genes (Figure 4). These findings are in line with the above findings that KLF2-AD-ID truncation mutant was a loss of function change, while KLF2-C274Y and H296Y mutants may be a gain-of-functional alteration.

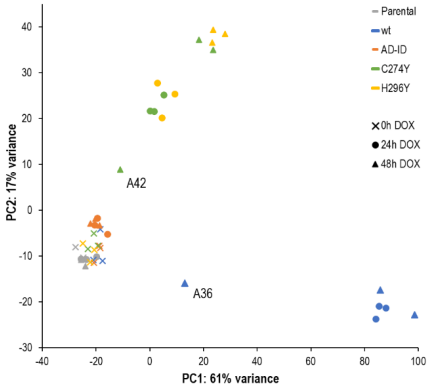

Figure 4. Principal component analysis. Samples expressing wild type KLF2 group together and samples expressing missense mutations group together (can also be further divided into 24h and 48h). All parental, AD-ID and 0h time points group together. Two outliers are highlighted, due to low KLF2 expression.

We next systematically compared the common and different gene expression among the KLF2-WT and mutants at both 24 and 48 hours induction in reference to controls, and subjected them to Gene Ontology analyses in order to identify the molecular function, cellular component, and biological processes potentially involved by KLF2 and its mutants. As expected, comparison between KLF2-AD-ID with controls yielded no or few but irrelevant Gene Ontology terms, while separate comparisons between KLF2-WT, missense mutants, and controls showed very similar results between samples at 24 and 48 hours induction for each condition. In light of these observations, the data analyses were subsequently focused on KLF2-WT and missense mutants with the experimental data at 24 and 48 hours induction combined together for each condition.

Independent analyses of common and different expressed genes between KLF2-WT and each of the missense mutants showed very similar results. Based on the common gene sets up and down-regulated by both KLF2-WT and missense mutants in reference to controls, the Gene Ontology terms unravelled included B-cell differentiation, NF-κB, NOTCH and WNT signalling, RNA processing or spliceosome machinery. Separate analyses based on the differentially expressed genes between KLF2-WT and its missense mutants revealed that the most prominent Gene Ontology terms was WNT signalling. These observations from global and hypothesis free analyses were consistent with the speculated role of KFL2 in regulation of follicular B-cell to marginal zone differentiation by previous knockout mice study.

To further validate the above observations, we performed GSEA analysis of the molecular pathways relevant to B-cell biology, comparing KLF-WT, missense mutants with reference controls. The results showed both KLF2 and its missense mutants were significantly involved in several common molecular pathways, including BCR, TLR, WNT, NOCTCH, chemokine, MAPK and JAK-STAT signalling. Importantly, both missense mutants appeared to have more positive enrichment in gene expression in these molecular pathways than the KLF2-WT. We next carried out GSEA analysis of the gene targets of transcriptional factors activated by the above signalling pathways, comparing KLF-WT, missense mutants with reference controls. Again, the results

demonstrated that both KLF2-WT and its missense mutants significantly affect the expression of the gene targets of several transcriptional factor.

Conclusions:

1) KLF2 also negatively regulates transactivation by NOTCH2 and MAPK/ERK signalling, thus most likely functioning as broad transcriptional repressor;
2) KLF2 truncation mutants lose the transcriptional repressor activities of KLF2 wild type, while some of the KLF2 missense mutants show gain of function, and predominantly localisation in nuclear speckles.
3) KLF2 regulates B-cell differentiation, NF-κB, NOTCH and WNT signalling through controlling gene transcription and possibly also RNA processing.

How Closely Have the Original Aims been Met:

We have achieved the objectives set up in the research proposal.

Outputs (including meeting abstracts, oral presentations, original papers, review articles) from the study in which the Pathological Society has been acknowledged:

We have presented our research in the following meetings.

Maria Rust, Ming Wang, Jovia Gao, Francesco Cucco, Alexandra Clipson, Ming-Qing Du. KLF2 is a global regulator of NOTCH2 and NF-κB and mutation abolishes its repressor activities. **Cambridge Lymphoma Biology International Symposium.** 17-18 July 2018; Poster presentation

Rust M, Wang M, Gao J, Cucco F, Clipson A, Chen Z, **Du MQ**. KLF2 is a Global Regulator of NOTCH2 and NF-κB and Mutation Abolishes its Repressor Activities. PathSoc 2020 Winter meeting, **Oral presentation**

We are in process of a manuscript preparation, aiming to get it submitted by end of 2021.