FOXO4 is Required for the Promotion of CLL Growth and Survival

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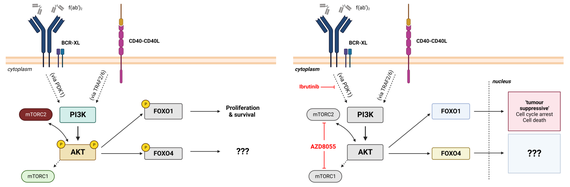
Introduction

Downstream of B-Cell Receptor (BCR)/CD40 signalling, the PI3K-AKT-mTOR axis promotes cell survival and proliferation via negative regulation of FOXO proteins. FOXOs are transcription factors belonging to the Forkhead Box (FOX) superfamily typically described as canonical 'tumour suppressors' due to their roles in pro-apoptotic and anti-proliferative responses.

We have previously demonstrated that synergistic inhibition of BCR ligation using AZD8055 (dual mTOR inhibitor) and Ibrutinib (BTKi) leads to CLL cell death coinciding with increased FOXO1 activity (Cosimo *et al*., 2019), thereby demonstrating FOXO1's tumour suppressive capability. However, unlike other B-cell malignancies, wider FOXO family function in CLL is poorly described (Lees *et al*., 2023). Here we describe a potential role for FOXO4 in CLL proliferation and survival, focusing on an shRNA-based approach to investigate the characteristics of FOXO4, as well as exploring FOXO4 expression and localisation in distinct CLL primary and cell line models.



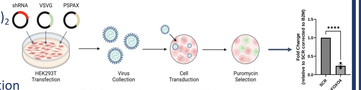
Normal conditions (NDC) — BTKi/mTORi treatment

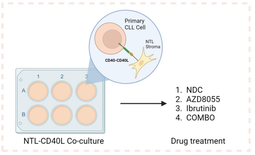
Objectives

To provide insight into novel FOXO4-mediated regulation of intracellular mechanisms following *in vitro* stimulation, drug treatment and shRNA-mediated *FOXO4* depletion.

Methods

1. NTL-CD40L *in vitro* co-culture, F(ab')$_2$ stimulation
2. Western blotting (WB)
   - *Ex vivo* samples, 1hr, 48hr treatments & cellular fractionation
3. RNA Sequencing
   - 5 patient samples, 24hr treatments
2. RT-qPCR
   - *FOXO4, BCL2L11, SESN3, GADD45A expression* (24 hr), cell line & patient samples
3. shRNA-mediated *FOXO4* knockdown (KD)
   - Cell lines & patient samples
4. Flow cytometry
   - CTV, Annexin/7-AAD, i/c pH2AX





NTL-CD40L primary CLL co-culture

Results

1 FOXO4 is differentially expressed in CLL cells

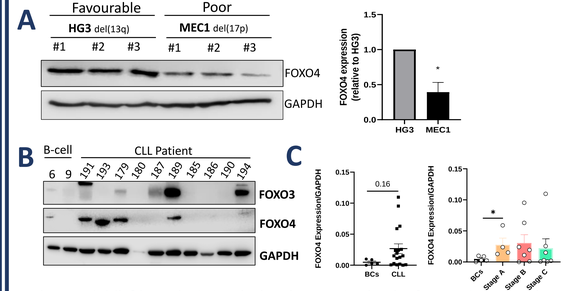


Figure 1. (A) (left) WB depicting the basal expression levels of FOXO4 in HG3 and MEC1 cell lines, which possess cytogenetic alterations associated with either favourable or poor prognosis respectively. (right) displays quantified intensity levels of FOXO4 expression between the two cell lines (n=3). (B) Protein lysates were prepared from *ex vivo* PB CLL patient samples and were processed via WB to detect FOXO4 expression from a wider cohort of patients (n=18) *vs.* healthy B-cell donors (n=5). The blots show differential FOXO4 expression, with a significant increase of FOXO4 expression seen within a Binet Stage A subgroup of CLL patients (A. n=4, B. n=7, C. n=7)

2 Stimulation promotes increased FOXO4 expression and further nuclear localisation

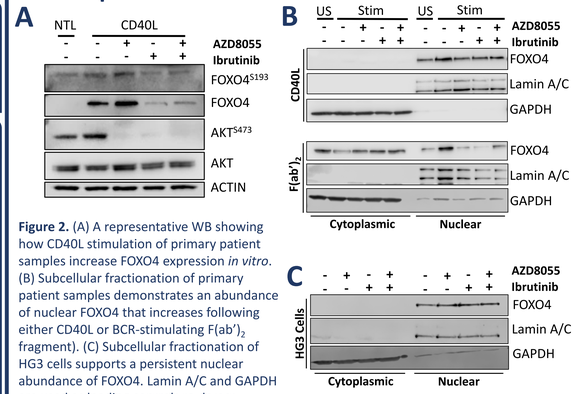
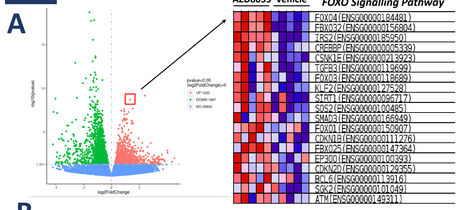


Figure 2. (A) A representative WB showing how CD40L stimulation of primary patient samples increase FOXO4 expression *in vitro*. (B) Subcellular fractionation of primary patient samples demonstrates an abundance of nuclear FOXO4 that increases following either CD40L or BCR-stimulating F(ab')$_2$ fragment). (C) Subcellular fractionation of HG3 cells supports a persistent nuclear abundance of FOXO4. Lamin A/C and GAPDH are used as loading controls to detect sufficient fractionation of nuclear and cytoplasmic fractions respectively.

3 FOXO4 expression is tightly controlled during CLL cell proliferation



B

Condition	Regulation	padj
CD40L stimulation	Down	$1.7x10^{-6}$
AZD8055	Up	$8.9x10^{-7}$
Ibrutinib	Up	n.s
AZD8055+Ibrutinib	Up	$1.7x10^{-6}$

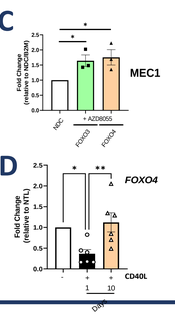
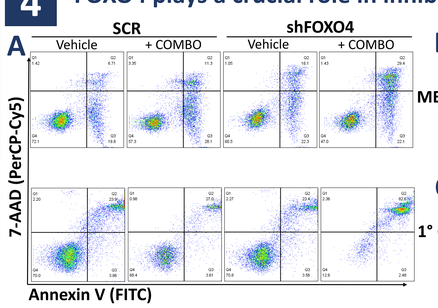


Figure 3. (A) (left) Volcano plot representing differentially expressed genes in an RNA-Seq dataset profiling 5 proliferating patient samples (+CD40L) in response to AZD8055 treatment. (right) A heatmap describing regulation changes within members of the FOXO signalling pathway with *FOXO4* being the most significantly upregulated. (B) Changes in *FOXO4* expression in response to CD40L stimulation, AZD8055, ibrutinib or combination treatment (n=5, 24 hr). (C) Validation in MEC1 cells mimics *FOXO* expression increases seen in the RNA-Seq dataset +AZD8055 (n=3). (D) RT-qPCR reveals a reduction in *FOXO4* expression (+CD40L) in primary cells that significantly increases following 10 days in *in vitro* co-culture (n=6).

4 FOXO4 plays a crucial role in inhibiting CLL chemosensitivity



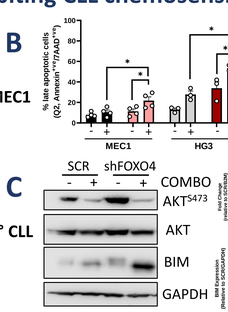
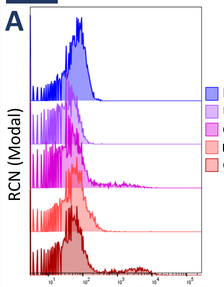


Figure 4. (A) Scatter plots depicting increased cell death in MEC1 (top) and primary CLL (bottom) cells with *FOXO4* KD following AZD8055-Ibrutinib combination treatments. (B) Significant increases of Annexin^{+}/7AAD^{+} MEC1 and HG3 *FOXO4*-KD cell populations following drug treatment (n=4 MEC1, n=3 HG3). (C) qPCR and WB analysis of the proapoptotic FOXO target gene *BCL2L11* and its protein product BIM, supporting an increase in apoptotic cellular environment following *FOXO4* KD and AZD8055-Ibrutinib treatment (MEC1 cells, n=4 qPCR, n=3 WB).

5 CLL cells are more susceptible to DNA damage following *FOXO4* KD



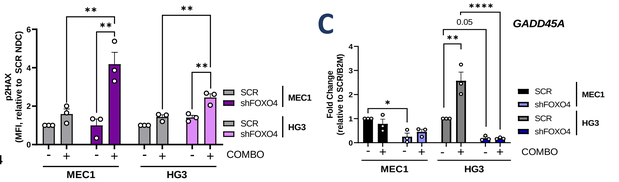
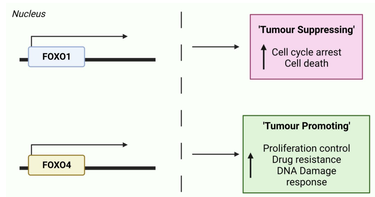


Figure 4. MEC1 and HG3 shFOXO4 & SCR control cells were cultured for up to 48 hr (A-C) in the presence of AZD8055 & Ibrutinib or vehicle control. Cells were then stained with pH2AX conjugated antibody and assessed via flow cytometry. (A) Representative histograms displaying pH2AX levels in SCR and shFOXO4 MEC1 cells including an isotype control. (B) Quantitative analysis of pH2AX intensity (MFI) in MEC1 and HG3 cell lines demonstrating increased pH2AX formation in drug treated shFOXO4 conditions. (C) RT-qPCR displays significant reductions in *GADD45A* expression in *FOXO4*-depleted MEC1 and HG3 cells (n=3).

Conclusions

- High nuclear abundance of FOXO4 and localisation in stimulated patient CLL cells points toward basally 'active' FOXO4 *in vitro*, compared with FOXO1 in CLL being 'inactive' (Cosimo *et al*., 2019).
- Significant changes in *FOXO4* expression in response to AZD8055-Ibrutinib treatments as well as throughout CD40L co-culture demonstrates how CLL survival and proliferation is partnered with discrete *FOXO4* regulation
- Increases in CLL cell chemosensitivity following shRNA-mediated *FOXO4* depletion suggests a novel requirement for FOXO4 abundance to promote CLL proliferation and survival.
- Varied levels of FOXO4 in distinct CLL subgroups demonstrates a potential link between FOXO expression and disease prognosis



References

Cosimo *et al.* (2019): AKT/mTORC2 Inhibition Activates FOXO1 function in CLL Cells Reducing B-Cell Receptor-Mediated Survival. *Clin Cancer Res.* doi: 10.1158/1078-0432.CCR-18-2036

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