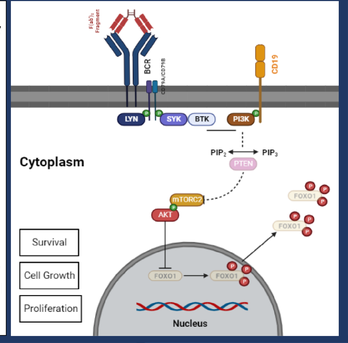
BCR-mediated Signals Modulate Rapid FOXO1 Upregulation in CLL: A Role for Deubiquitinase Proteins?

Hassan N. B. Almuhanna, Jodie Hay, Michael W. Moles, Alison M. Michie
Paul O'Gorman Leukaemia Research Centre, School of Cancer Sciences, University of Glasgow, Glasgow G12 8QQ, UK

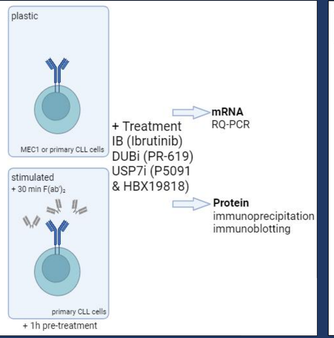
Friends of the Paul O'Gorman Leukaemia Research Centre Glasgow
University of Glasgow
PCR BIOSYSTEMS simplifying research
UKCLL FORUM FINDING A CURE TOGETHER
Mohammed Al-Mana College for Medical Sciences
Ministry of Education
Saudi Arabian Cultural Bureau in the UK
PRINTED BY SCIENCEPOSTERS .co.uk

Introduction

The transcription factor Forkhead box protein, subgroup O-1 (FOXO1) is inactivated downstream of BCR activation. We previously demonstrated that FOXO1 activation regulates chemosensitivity and induces apoptosis (1). Here we show that FOXO1 expression is rapidly upregulated upon BCR crosslinking and reversed with ibrutinib (IB) treatment. These findings prompted us to investigate whether deubiquitinase (DUB) proteins regulate FOXO1 expression/activity in CLL cells, with a view to identifying potential novel drug targets that could enhance current CLL treatments. Our analysis revealed a significant upregulation of expression of specific DUB family members in freshly isolated CLL cells compared to healthy B-cells. Treatment of the CLL cell line MEC1 and primary CLL patient samples with DUB inhibitors alone reduced phosphorylation of AKT^{S437} and FOXO1^{T24}, indicating that DUBs play a role in inhibiting FOXO1 activity. We demonstrated a direct interaction between FOXO1 and USP7, which was enhanced upon BCR crosslinking. Furthermore, the expression of FOXO1 gene targets was altered in USP7 shRNA Knockdown (KD) cells. Analysis of FOXO1 localisation showed significant upregulation of FOXO1 in the nuclear fraction upon IB treatment, which was further enhanced in the USP7 KD cells, or the combination with DUBi. Indeed, USP7 KD sensitised MEC1 cells to IB treatment leading to increase in FOXO1 nuclear localisation. Additionally, FOXO1 activity, which was elevated upon IB treatment, was further enhanced in the USP7 KD cells or combination with a selective USP7i. These studies indicate that selective DUBs play a role in BCR-mediated signalling to aid in the promotion of CLL cell survival and chemo-resistance through FOXO1 inhibition, and targeting these DUBs enhance FOXO1 activity leading to cell cycle arrest and apoptosis.

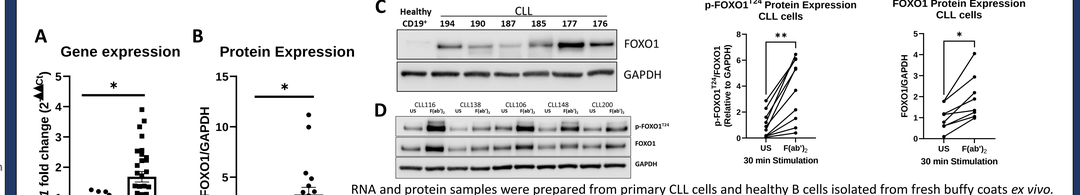


Methods



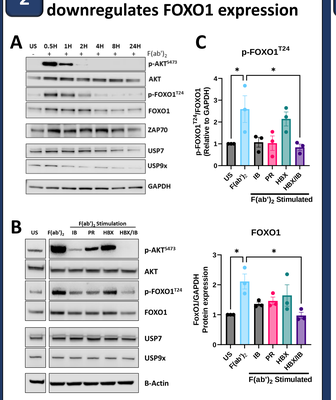
Results

1 FOXO1 expression is rapidly upregulated upon BCR ligation



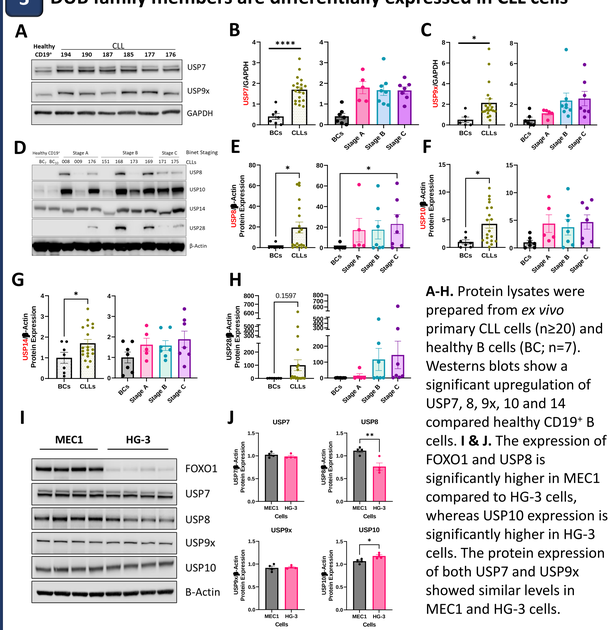
RNA and protein samples were prepared from primary CLL cells and healthy B cells isolated from fresh buffy coats *ex vivo*. **A.** RT-qPCR revealed a significant upregulation expression of *FOXO1* in primary CLL cells (n=30) compared to healthy CD19$^+$ cells (n=9). **B&C.** Western blots show a significant upregulation of FOXO1 in primary CLL cells (n=18) compared to healthy CD19$^+$ cells (n=8). **D–F.** Primary CLL cells (n≥8) were incubated with 10 µg/ml F(ab')$_2$ for 30 min. Immunoblots for p-FOXO1^{T24} and FOXO1 show a significant upregulation of p-FOXO1^{T24} and FOXO1 upon BCR crosslinking compared with unstimulated (US) cells.

2 BCR- and DUB-signalling inhibition downregulates FOXO1 expression



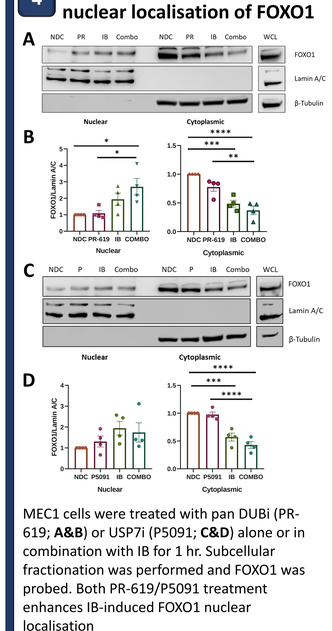
A. CLL protein lysates were immunoblotted for p-FOXO1^{T24}, FOXO1, USP7 and USP9X, p-AKT^{S473}, AKT and ZAP70 after BCR crosslinking for up to 24 hr as indicated. FOXO1 expression was rapidly elevated upon BCR crosslinking. **B & C.** IB treatment downregulated p-FOXO1^{T24} and FOXO1 expression, and IB/USP7i combination treatment further enhanced FOXO1 downregulation.

3 DUB family members are differentially expressed in CLL cells



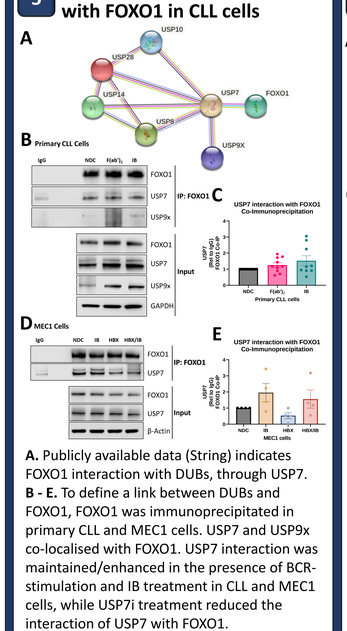
A-H. Protein lysates were prepared from *ex vivo* primary CLL cells (n≥20) and healthy B cells (BC; n=7). Westerns blots show a significant upregulation of USP7, 8, 9x, 10 and 14 compared healthy CD19$^+$ B cells. **I & J.** The expression of FOXO1 and USP8 is significantly higher in MEC1 compared to HG-3 cells, whereas USP10 expression is significantly higher in HG-3 cells. The protein expression of both USP7 and USP9x showed similar levels in MEC1 and HG-3 cells.

4 DUB inhibition enhances nuclear localisation of FOXO1



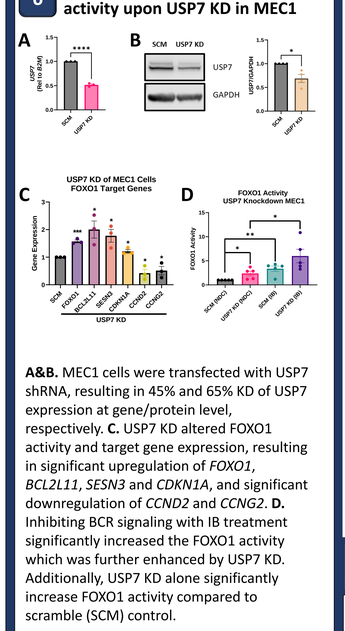
MEC1 cells were treated with pan DUBi (PR-619; **A&B**) or USP7i (P5091; **C&D**) alone or in combination with IB for 1 hr. Subcellular fractionation was performed and FOXO1 was probed. Both PR-619/P5091 treatment enhances IB-induced FOXO1 nuclear localisation

5 USP7 protein directly interacts with FOXO1 in CLL cells



A. Publicly available data (String) indicates FOXO1 interaction with DUBs, through USP7. **B - E.** To define a link between DUBs and FOXO1, FOXO1 was immunoprecipitated in primary CLL and MEC1 cells. USP7 and USP9x co-localised with FOXO1. USP7 interaction was maintained/enhanced in the presence of BCR-stimulation and IB treatment in CLL and MEC1 cells, while USP7i treatment reduced the interaction of USP7 with FOXO1.

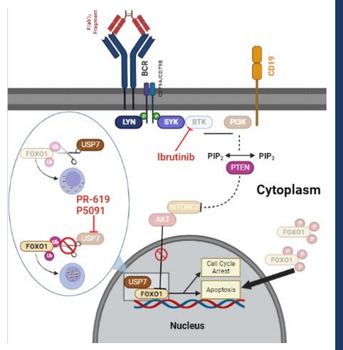
6 Significant increase in FOXO1 activity upon USP7 KD in MEC1



A&B. MEC1 cells were transfected with USP7 shRNA, resulting in 45% and 65% KD of USP7 expression at gene/protein level, respectively. **C.** USP7 KD altered FOXO1 activity and target gene expression, resulting in significant upregulation of *FOXO1*, *BCL2L11*, *SESN3* and *CDKN1A*, and significant downregulation of *CCND2* and *CCNG2*. **D.** Inhibiting BCR signaling with IB treatment significantly increased the FOXO1 activity which was further enhanced by USP7 KD. Additionally, USP7 KD alone significantly increase FOXO1 activity compared to scramble (SCM) control.

Summary:

- BCR-crosslinking promotes rapid upregulation of FOXO1 and ZAP70 expression in CLL cells.
- FOXO1 upregulation is inhibited by IB treatment, and partially inhibited by DUBi, suggesting a dependence of FOXO1 expression on BCR signaling.
- Combination of USP7i and IB significantly downregulated p-AKT^{S473}, p-FOXO1^{T24} and total FOXO1, enhancing the effect of IB on FOXO1 regulation.
- Specific DUB family members are differentially regulated in CLL cells compared to healthy controls, as well as MEC1 and HG-3 cells.
- FOXO1 colocalizes with USP7, and a significant correlation exists between the expression of FOXO1 and USP7.
- IB induces nuclear localization of FOXO1 which is enhanced with DUBi: PR-619 or P5091 (USP7i)
- FOXO1 and USP7 co-localize which suggests that DUB proteins may be key regulators of FOXO1 activity.
- USP7 shRNA KD in MEC1 cells altered expression of known FOXO1 target genes and significantly increases FOXO1 DNA binding activity, which was further enhanced with IB treatment.



Conclusion:

These data indicate that USP7 inhibition maintains FOXO1 nuclear localization, thus enhancing FOXO1 activation and subsequent CLL cell death.

Reference

1. Cosimo, *et al*. AKT/mTORC2 inhibition activates FOXO1 function in CLL cells reducing B-cell receptor-mediated survival. *Clinical Cancer Research* 25.5 (2019): 1574-1587.