**Abstract**

Diffuse large B-cell lymphoma (DLBCL) accounts for up to 40% of non-Hodgkin lymphoma and there are few therapeutic options for patients who don’t respond to the standard of care. The transcription factor FOXO1 is recurrently mutated (mFOXO1) across DLBCL subtypes and enriched in relapsed/refractory patients. Unlike other tumor types, DLBCL mFOXO1 mutations appear to be activating and disrupt negative regulatory function of FOXO1 by AKT. While deletion of Foxo1 in mice demonstrated it is required for proper B-cell development, the consequences of putative activating mutations have not been investigated. Furthermore, genetic deletion studies cannot distinguish between direct and indirect transcriptional effects because they require days or weeks before analysis can begin. Prior studies determining the mechanisms by which FOXO1 controls gene expression are fragmented, limited in scope, and the results are contradictory and cell type specific.

To overcome the limitations posed by traditional techniques, we have paired the condition-tag (dTAG) system with the dTAG system to study the biological and transcriptional effects of FOXO1 degradation in the context of DLBCL. We have engineered the endogenous locus for WT (SU-DHL-4) and mFOXO1 (OCI-Ly1) (Fig 1B). These cell lines allowed us to rapidly degrade the endogenous FOXO1-FKBP12-H2O2 protein to study the direct effects of FOXO1 loss in these cells. FOXO1 protein levels are drastically decreased within 0.5 hr of dTAG-47 treatment and gene expression starts to recover by 2 hrs. This recovery is not seen in the OCI-LY1 (mut) cells. Fewer genes overlap between time points for significantly downregulated genes in SU-DHL-4 (WT) cells (Fig 2A). This finding could point to a mechanism in which WT FOXO1 is dispensable for the maintenance of a gene expression program that cells are no longer able to maintain after degradation.

Using the clusters from the PRO-seq analysis, we looked at the changes in the mRNA levels by 6 hrs (green) in the SU-DHL-4 (WT) cells. This system allows us to rapidly degrade FOXO1 with the dTAG system in a transient manner to study the direct and indirect transcriptional effects of FOXO1. We employed this system to study the biological and transcriptional effects of FOXO1 degradation in the context of DLBCL. We have engineered the endogenous locus for WT (SU-DHL-4) and mFOXO1 (OCI-Ly1) (Fig 1B). These cell lines allowed us to rapidly degrade the endogenous FOXO1-FKBP12-H2O2 protein to study the direct effects of FOXO1 loss in these cells. FOXO1 protein levels are drastically decreased within 0.5 hr of dTAG-47 treatment and gene expression starts to recover by 2 hrs. This recovery is not seen in the OCI-LY1 (mut) cells. Fewer genes overlap between time points for significantly downregulated genes in SU-DHL-4 (WT) cells (Fig 2A). This finding could point to a mechanism in which WT FOXO1 is dispensable for the maintenance of a gene expression program that cells are no longer able to maintain after degradation.

To determine if nascent transcriptional changes correlated with changes at the mRNA level we performed an RNA-seq time course (Fig 2C) after a time course of dTAG-47 treatment (0, 0.5, 1, and 2 hrs after dTAG-47 treatment). FOXO1 degradation in our cell line models cause rapid transcriptional changes within 0.5 hr of treatment. Most significantly changed genes (±1.5 fold change and padj ≤ 0.05) were downregulated and a large portion of these changes could be seen within 0.5 hr of dTAG-47 treatment (Fig 3A/B). A considerable number of downregulated genes overlapped between at least 2 time points (Fig 3C/D), indicating that these genes are direct targets of FOXO1. However, only a handful of upregulated genes overlapped between time points (Fig 3E/F). These transcriptional changes correspond to the downregulation of genes related to the germinal center (GC) light and dark zone (DZ) (Fig 3G/H) which recapitulates gene expression profiles of Fox1 in GC B cells in vivo.

To determine if nascent transcriptional changes correlated with changes at the mRNA level we performed an RNA-seq time course (0, 6, and 24 hrs after dTAG-47 treatment). While we saw rapid nascent transcriptional changes after FOXO1 degradation in both cell lines, these only overlapped with changes associated with genes downregulated in the OCI-Ly1 (mut) cell line. RNA-seq analysis showed that there were fewer changes in gene expression in the SU-DHL-4 (WT) line than the OCI-Ly1 (mut) line (Fig 4A). In addition to fewer overall changes in gene expression, the percentage of downregulated genes that overlapped between RNA-seq time points was much lower in the SU-DHL-4 (WT) line (Fig 4C/D). Genes downregulated in any RNA-seq time point were also less likely to overlap with genes downregulated in any PRO-seq time point in SU-DHL-4 (WT) line (Fig 4F/G).

To assess whether nascent transcriptional changes were not translating into changes in mRNA levels, we clustered the genes that were significantly regulated in at least two time points (each data set was clustered independently). We identified clusters of genes in both data sets that start to return to base line by two hours. This cluster (cluster 3) is much larger in SU-DHL-4 (WT) than OCI-Ly1 (mut) cell lines and shows that the gene expression significantly decreases by 0.5 hr. However, cluster 3 genes are not significantly decreased by 0.5 hrs. Further analysis of the PRO-seq and RNA-seq data showed that the expression levels of these genes were significantly decreased in SU-DHL-4 (WT) cells, however the majority of these genes were not significantly changed in OCI-Ly1 (mut) cells (Fig 5A). This finding could point to a mechanism in which WT FOXO1 is dispensable for the maintenance of a gene expression program that cells are no longer able to maintain without hyperactive mFOXO1.

**Conclusion**

- Degradation of mFOXO1 impairs cell growth and causes an accumulation in G1/G0.
- FOXO1 primarily activates transcription in OCI-LY1 cells.
- FOXO1 degradation causes downregulation of dark zone genes indicating that our FOXO1- knockdown lines are faithful models of FOXO1 function.
- FOXO1 degradation results in gene expression changes at the mRNA level, but WT FOXO1 degradation does not cause changes.
- WT FOXO1 gene expression changes are transient and start to return to baseline by 2 hrs post degradation, however, mFOXO1 mediated gene expression changes are sustained.

**Future Directions**

- Determine FOXO1 localization in both cell lines (CUT&RUN<sup>C/PRO-seq</sup>)
- Measure changes in H3K27ac at FOXO1 regulated genes (Chipseq)
- Analyze the effects of FOXO1<sup>R21P</sup> on GC formation and GC dynamics in vivo
- Assess tumor incidence, prevalence, and severity in Cry1-cre;Plk6<sup>-/-</sup> or Tgβ02<sup>+</sup>; Fox1<sup>R21P</sup> mouse models

**References**


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