PAX3-FOXO1 contributes to aRMS tumorigenesis through transcriptional activation of its targets to inhibit myogenic differentiation

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ABSTRACT

Master regulatory transcription factors are disrupted by chromosomal translocations in leukemia and solid tumors. Rhabdomyosarcoma (RMS) is one of the most common solid tumors in children, accounting for approximately 30% of all soft tissue sarcomas. One of the major subtypes of RMS, alveolar rhabdomyosarcoma (aRMS) cases have a relatively low 5-year overall survival, with a high chance of metastasis and relapse. The t(2;13) (PAX3-FOXO1) fusion gene is a signature genetic change of pediatric aRMS, and the resulting fusion protein acts as a novel transcription factor which we sought to be the main cause of aRMS. One of the challenges to studying transcription factors is that it is difficult to rapidly inhibit their function to access the immediate targets. Here we modified the endogenous aRMS cell line to express a PAX3-FOXO1-FKBP12 fusion protein linked to the PROTAC to rapidly degrade the endogenous PAX3-FOXO1. Our data show that losing of this fusion protein impaired cell proliferation and induced cell differentiation. The expression of two factors involved in muscle differentiation, myogenin and myosin heavy chain, were increased after PAX3-FOXO1 degradation. Moreover, PAX3-FOXO1 was required for anchorage-independent growth which is hallmark of carcinogenesis for transformed cells. In addition, we used the cleavage under targets and release using nuclease (CUT&RUN) to map PAX3-FOXO1 binding sites with exceptionally low background levels, and to verify the binding sites by comparing the signal before and after degradation PAX3-FOXO1. Furthermore, we combined rapid PAX3-FOXO1 degradation with precision nuclear run-on transcription coupled with deep sequencing (PRO-seq) and found that there were 127 genes that were down regulated in gene body, while only 32 genes with up regulated gene body change, suggesting that PAX3-FOXO1 function mainly as an activator to transcription regulation. Finally, with the thorough profiling study, we identified a list of high-confidence targets that regulated by PAX3-FOXO1, including transcription factors, cofactors, signaling proteins. In conclusion, rapid degradation of endogenous PAX3-FOXO1 not only characterizes the mechanisms of PAX3-FOXO1 driven tumorigenesis, but also provides insights into potential therapeutic benefits to PAX3-FOXO1 harbored RMS cases.

Inhibition of PAX3-FOXO1 affects aRMS cell proliferation

Figure 1: Degradation of PAX3-FOXO1-HA-FKBP12 fusion protein in t(2;13) aRMS cell line (Rh30) and inhibition of PAX3-FOXO1 reduces aRMS cell proliferation, induces cell cycle arrest, and reduces tumorigenic growth. A. The construction of donor plasmid DNA template we used to insert FKBP12/F36V-P2A-mCherry into the endogenous PAX3-FOXO1 alleles. B. Schematic depiction of the dTAG strategy. The derivative of thalidomide analog dTAG7 binds to the FKBP module to link PAX3-FOXO1-FKBP to the cereblon E3 ligase to cause rapid degradation of PAX3-FOXO1-FKBP. C. Western blot analysis of Rh30, PAX3-FOXO1-FKBP cells treated with or without the indicated times (Kristy Stengel). D. Rh30 PAX3-FOXO1-FKBP cells were treated with 500 nM dTAG47, and cell counts were determined using a Becton-Dickinson flow cytometer. Data are mean ± SEM (n=3). E. Rh30, PAX3-FOXO1-FKBP cells were treated with 500 nM dTAG47 for the indicated times. Flow cytometry plots of incorporated BrdU versus PI show the decreasing of cells in S-phase and accumulation of cells in G1-phase. F. Bar graph shows the percentage of cells in S-phase and G1-phase. G. Shown are representative images of colony formation. The bottom bar graph displays the counts of colonies at 4X. Data are mean ± SEM (n=9). **P<0.001 by mann-whitney test.

Inhibition of PAX3-FOXO1 induces myogenic differentiation

Figure 3: Inhibition of PAX3-FOXO1 induces myogenic differentiation in aRMS cells. A. Immunofluorescence staining of Myosin Heavy Chain (A) and myogenin (B). Rh30 PAX3-FOXO1-FKBP cells were treated with 500 nM dTAG47 for 6 days. DAPI was used to label nuclei (blue). Alexa 568 labeled Phalloidin was used to mark actin filaments (red). Alexa 488 secondary antibody was used to visualize the primary antibody against Myosin Heavy Chain and myogenin (green). Fluorescence was visualized with a super-resolution fluorescent microscope (20X).

Identification of PAX3-FOXO1 binding sites in genome of aRMS cells

Figure 4: Mapping of PAX3-FOXO1 binding site in aRMS cells. A. Comparison of heatmaps of CUT&RUN anti-HA PAX3-FOXO1 peak signal intensity +/- 1.5 kb from peak center in the presence (left) and absence (right) of endogenous PAX3-FOXO1. B. Annotation of PAX3-FOXO1 peak indicated most PAX3-FOXO1 binding sites are intergenic or intronic regulatory elements, and 20% are at gene promoters. C. Nucleotide logo of the predicted PAX3-FOXO1 binding sites.

Figure 5: PRO-seq analysis reveals that PAX3-FOXO1 causes both transcription activation and repression of target genes. A. PRO-seq analysis reveals that PAX3-FOXO1 causes both transcription activation and repression of target genes. A. PRO-seq analysis reveals that PAX3-FOXO1 causes both transcription activation and repression of target genes. B. Heatmaps show the increased (left) and decreased (right) gene body change after degrading endogenous PAX3-FOXO1 at indicated timepoints. C. Heatmaps show the increased (left) and decreased (right) gene body change after degrading endogenous PAX3-FOXO1 at indicated timepoints.

High-confidence targets regulated by PAX3-FOXO1 in aRMS cells

Figure 6: Combination of the analysis of PRO-seq, RNA-seq, and CUT&RUN identified a short list of high-confidence targets regulated by PAX3-FOXO1 in aRMS cells. A. Venn diagram reveals 79 overlapped genes are significantly changed among all the three profiling experiments. B. Expression heatmap the relative transcriptional expression levels of the 79 genes from 0-72 hours following degradation of endogenous PAX3-FOXO1. Data were plotted from RNA-seq after the Z-score normalization across the row. The colors vary from blue to red representing the scale of the relative expression levels. C. CUT&RUN signal and PRO-seq (anti-sense transcription: yellow tracks, sense strand transcription: blue tracks) signal at representative loci. Green tracks are CUT&RUN signal before and after degradation of PAX3-FOXO1.

CONCLUSIONS & FUTURE DIRECTIONS

• CRISPR-based modification of the endogenous PAX3-FOXO1 locus allows rapid degradation of the transcriptional factor.
• Inhibition of PAX3-FOXO1 affects aRMS cell growth and induces myogenic proliferation.
• Combination of CUT&RUN and PRO-seq enables the identification of the direct targets of endogenous PAX3-FOXO1 in aRMS cells.
• Determination of the effects after inhibition of the identified targets of PAX3-FOXO1.
• Identification of the binding partners of PAX3-FOXO1 that involved in regulating the targets.

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