Role of *FOXR2* in malignant peripheral nerve sheath tumor progression

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Introduction

MPNSTs: Aggressive and difficult to treat sarcomas

- Malignant peripheral nerve sheath tumors (MPNSTs) originate from benign neurofibromas in the peripheral nervous system and frequently metastasize to the lungs and other tissues.
- Lifetime risk: **1 in 10,000** for the general popluation, but **1 in 10** for individuals with neurofibromatosis type 1 (NF1) syndrome.
- 5-year survival rate: **30%**
- Current treatment: Surgical removal (if possible) and chemotherapy.

How can we do better?

- Persistently low survival rate for patients with MPNSTs highlights the need for effective, targeted therapies.
- In order to approach new treatments, we need to develop a better understanding of these cancers on a molecular level.
- Insertional mutagenesis screens utilize mouse models of cancer to identify novel cancer-associated genes.

FOXR2: A novel driver of MPNST pathogenesis?

- Schwann cell-targeted *Sleeping Beauty* insertional mutagenesis screen in mice identified the transcription factor *Foxr2* as a novel oncogene associated with MPNSTs.
- *FOXR2* overexpression in normal Schwann cells induces proliferation and anchorage-independent colony formation.
- Knocking down *FOXR2* in MPNST-derived cell lines has the reverse effect – slower proliferation and decreased colony formation

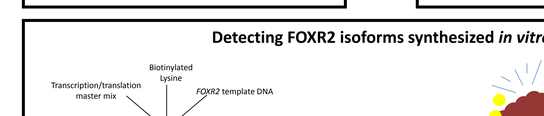


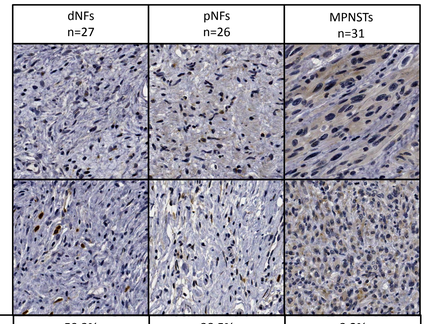
Figure 1 – *Diagram of transposon insertions near the* Foxr2 *gene in a mouse model of MPNST formation.* Position and orientation of arrowheads correspond to transpositions detected in mouse MPNSTs.

Characterizing FOXR2: Goals and Hypotheses

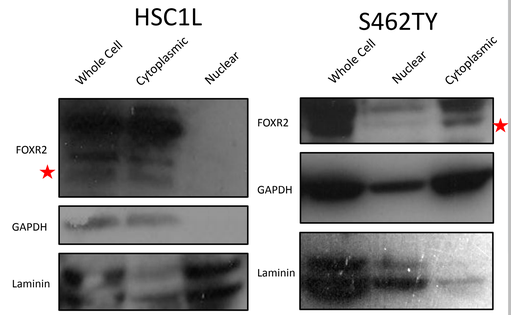
1. **Determine the subcellular localization of FOXR2.** Localization is a common method of regulating protein function. Hypothesis: Subcellular localization affects FOXR2 activity and may be altered in an oncogenic state.
2. **Evaluate low molecular weight FOXR2 isoforms.** Blotting for FOXR2 reveals full-sized and shorter versions of the protein. Hypothesis: Small FOXR2 isoforms are produced during translation rather than as a result of post-translational modification.
3. **Determine if FOXR2 is phosphorylated.** Bioinformatic analysis suggests several sites for phosphorylation. Hypothesis: FOXR2 is phosphorylated at one or more Serine residues.

Results

FOXR2 is located in the cytoplasm of Schwann cells



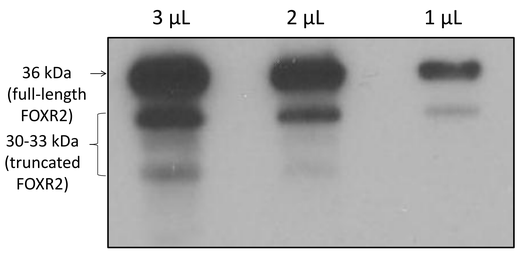
	dNFs n=27	pNFs n=26	MPNSTs n=31
Nuclear	59.3%	38.5%	3.2%
Cytoplasmic	0%	1.5%	67.7%
Both	29.6%	34.6%	12.9%
Negative	11.1%	15.4%	6.5%



★ **Additional observation:** FOXR2 is present as full-length (36 kDa) and shorter (30-33 kDa) isoforms.

Figure 2 – *FOXR2 localizes to the cytoplasm in transformed Schwann cells and is present in multiple isoforms.* IHC showing FOXR2 localization in dermal and plexiform neurofibromas (dNFs and pNFs, respectively) and MPNSTs (left). Immunoblot analysis was performed on cytoplasmic and nuclear fractions, as well as whole-cell lysates, from MPNST-derived S462TY cells and HSC1λ cells expressing a FOXR2-Flag fusion protein using the indicated antibodies (right).

FOXR2 forms truncated proteins *in vitro*



Potential translation start sites within the *FOXR2* reading frame

+1	...CCAGTAA**AAGATGG**ACTTA...	35.9 kDa
+76	...GACTGG**GAC*ATG***AGGAAT...	32.9 kDa
+160	...GTCGGA**GTAATG**AAGCCC...	29.8 kDa
+175	...CCCCCA**GAAATG**CCTCAG...	29.2 kDa
+238	...AATCTG**TGGATGT**GGGTG...	26.8 kDa
+514	...TCCCCT**GAAATG**AAATGT...	16.8 kDa
+895	...CAAGAG**TGCATGA**GTCAG...	1.5 kDa

Figure 3 – *FOXR2 is truncated* in vitro. Immunoblot against newly-synthesized biotinylated protein using the indicated volume of *in vitro* FOXR2 transcription and translation reaction mixture (left). Diagram of ATG sequences within the *FOXR2* gene (right). The Kozak sequence is highlighted with the color corresponding to either optimal (green), acceptable (yellow) or suboptimal (red) agreement with the consensus sequence. The predicted molecular weight of each fragment is shown.

No evidence of FOXR2 phosphorylation

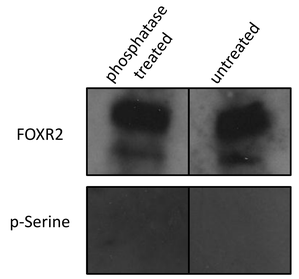
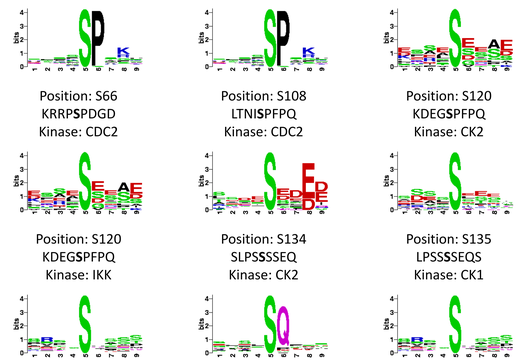
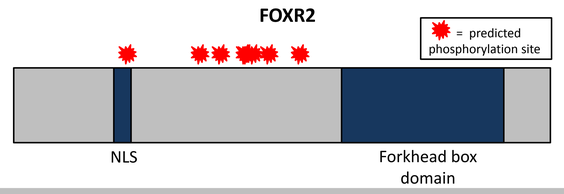
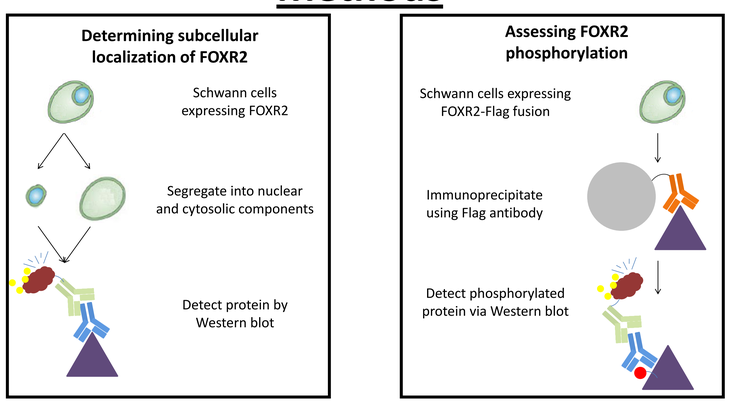


Figure 4 – *Phosphoserine antibody fails to detect phospho-FOXR2.* Immunoblot detecting either FOXR2 or phosphorylated serine in the 36 kDa region from Flag-precipitated HSC1λ *FOXR2:Flag* lysate pre-treated or untreated with phosphatase (left). Predicted phosphorylation targets within the FOXR2 protein with respective amino acid sequences and targeting kinases (middle). Diagram of FOXR2 with predicted phosphorylation sites marked (bottom).





Methods



Conclusions and Future Directions

Major Findings

1. **Cytoplasmic localization is sufficient – or possibly required – for FOXR2-mediated transformation.** MPNSTs express more FOXR2 than do benign neurofibromas, with much greater proportion of FOXR2 located in the cytoplasm in malignant tumors. Cells that have been transformed by FOXR2 overexpression seem to express FOXR2 exclusively in the cytoplasm.
2. **Full-length and truncated FOXR2 isoforms are the result of alternative translation start sites.** *In vitro* translation data indicate that FOXR2 truncation is not the result of post-translational modification. Rather, downstream ATG sequences are used as alternative translation start sites, resulting in N-terminal truncations.
3. **No evidence of FOXR2 phosphorylation thus far.** Despite strong bioinformatic evidence, we were unable to detect phosphorylated FOXR2. A more robust analysis, including examining protein from multiple cell lines, may provide a more definitive answer. Detection of radiolabelled phosphate may be a useful alternative to detection using phosphoserine-targeted antibodies.

Looking Forward

- **What does FOXR2 regulate?** – Our lab has carried out RNA transcriptome sequencing on cells expressing basal and elevated levels of FOXR2 to determine genes whose expression is altered by FOXR2. This will give us an indication of the mechanism by which FOXR2 promotes oncogenesis.
- **How is FOXR2 localization controlled?** – Despite being a transcription factor, FOXR2 appears to have an oncogenic effect even (or perhaps only) when it is restricted to the cytoplasm. A better understanding of this regulatory mechanism could shed light on the role of *FOXR2* in oncogenesis.
- **What is the significance of FOXR2 truncation?** – Does the truncated FOXR2 protein have altered function? Elevated FOXR2 expression seems to favor the formation of the truncated product – does this contribute to the oncogenic effects of FOXR2?
- **Can FOXR2 be targeted for MPNST therapies?** – FOXR2-positive MPNSTs may be responsive to inhibition of FOXR2. If so, isoform-specific (truncated versus full-length) or cytoplasm-specific blockers may be of particular use in targeting cancer cells while sparing normal cells.