Mitchell-Riley Syndrome: Investigating the Molecular Basis of RFX6-Dependent Morphogenetic Events using the amphibian *Xenopus*

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Introduction: Mitchell-Riley Syndrome (MRS) is a rare congenital condition characterized by a range of severe birth defects of the gastrointestinal (GI) tract and associated organs, including duodenal atresia, intestinal malrotation, pancreatic agenesis/hypoplasia, and gallbladder agenesis/hypoplasia. MRS patients typically present with severe neonatal diabetes, low birth weight and failure to thrive, persisting feeding difficulties, steatorrhea, and liver failure. MRS is caused by mutations in the regulatory factor x6 (*RFX6*) gene, an evolutionarily conserved DNA-binding transcription factor known to regulate GI development in vertebrates. At a mechanistic level, it is unclear how mutated *RFX6* alleles, that result in its compromised protein function, lead to the wide range of GI defects in patients. This project examines the function of Rfx6 during normal GI development in the amphibian *Xenopus* (the African clawed frog), a widely used vertebrate animal model for studies of GI research and human birth defect/disease modeling. We hypothesize that intestinal malrotation and duodenal atresia observed in human MRS result from disruptions to a RFX6-dependent signaling cascade between the endoderm and mesoderm layers of the embryo during GI development.

Methods: RFX6 loss-of-function experiments were performed in *Xenopus laevis* embryos to investigate the molecular basis of GI birth defects observed in human MRS patients. Such experiments involved microinjection of a morpholino oligo that inhibited Rfx6 protein translation into embryos (obtained by in-vitro fertilization) at the 2- to 4-cell stage of development, thereby generating the experimental Rfx6 loss-of-function group. A control morpholino oligo was injected into embryos at the same stage of development, generating the control group. Gene or protein expression changes were assayed using RNA in-situ hybridization, immunofluorescence, and confocal microscopy. In parallel experiments, FGF and Hedgehog pathway loss-of-function experiments were performed in *Xenopus* by adding a pharmacological antagonist (FGF: PD173074, Hh: cyclopamine) of each pathway simply to the culture buffer within which the embryos developed. Effects on intestinal rotation were analyzed by brightfield stereomicroscopy and/or microdissection of the GI tract from embryos.

Results: We observed that Rfx6 loss-of-function Xenopus embryos displayed phenotypes similar to those observed in human MRS patients, including intestinal malrotation, stomach/duodenal hypoplasia, pancreatic hypoplasia, and gallbladder agenesis. Reduced expression of the important signaling factor sonic hedgehog (Shh) was observed in the GI tract endoderm of Rfx6-deficient Xenopus embryos.

Conclusions: RFX6 plays an evolutionarily conserved, essential role in proper development of both the GI tract endoderm and mesoderm during embryogenesis, wherein Rfx6 controls a Hedgehog- dependent signaling cascade between these two tissue layers. Moreover, our data suggest that disruptions to RFX6-dependent Hh or FGF signaling could partially explain many of the phenotypes observed in human Mitchell-Riley Syndrome patients harboring mutations in *RFX6.*

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