Conditional and Differential Ablation of *Crkl* Splice Variants Recapitulates Human CAKUT Phenotypes in Mouse Models of DiGeorge Syndrome

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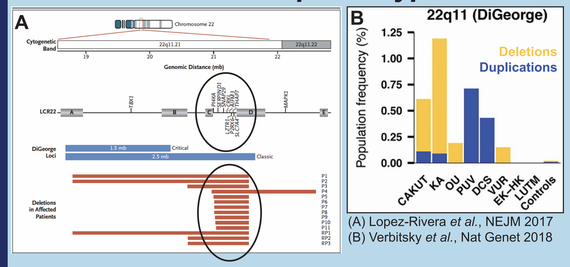
Introduction

Deletions in Chr 22q11.2 are associated with CAKUT phenotypes



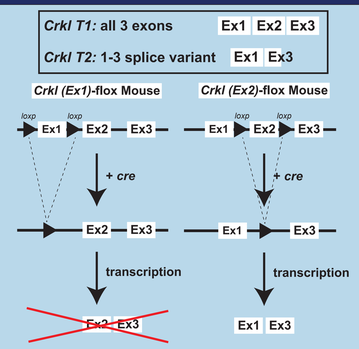
(A) Lopez-Rivera *et al.*, NEJM 2017
(B) Verbitsky *et al.*, Nat Genet 2018

Rationale: (A) Mutations in *CRKL* were previously identified as key drivers of CAKUT phenotypes in the DiGeorge, or 22q11.2, Syndrome (Lopez-Rivera et al., NEJM 2017).**(B)** At this locus, **deletions** were mostly identified in patients with kidney anomalies (KA) whereas **duplications** were associated with lower urinary tract defects, suggesting differential attributes of *CRKL* mutations and gene dosage for upper and lower anomalies of the urinary tract.

Hypothesis: *Crkl* splice variants can differentially regulate specific events of kidney and urinary tract development, either independently of one another or by modulating the activity or expression of eachother.

Objective: Here, we characterize the expression and developmental necessity of *Crkl* splice variants and isoform balance using conditional and global mutants.

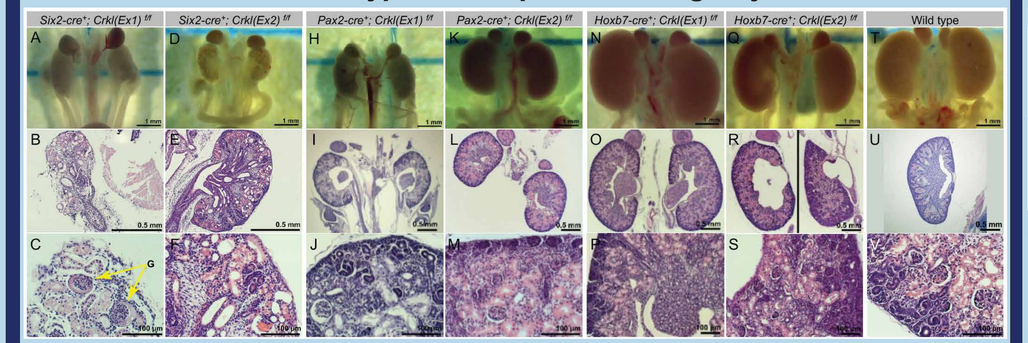
Methods



Conditional genetic recombination via *cre-lox* was used to drive the ablation of one (T1) or both (T1 + T2) *Crkl* splice variants in the metanephric mesenchyme (MM), both the MM and ureteric bud (UB), or from the UB alone by crossing floxed mutants with one of 3 cre mouse lines: *Six2-cre*, *Pax2-cre*, *Hoxb7-cre* (resp.). Mice missing one or both splice variants in the MM were analyzed for alterations in branched tissue morphogenesis via IHC, and RNAseq-based differential gene expression at 13.5dpc and 15.5dpc.

Results

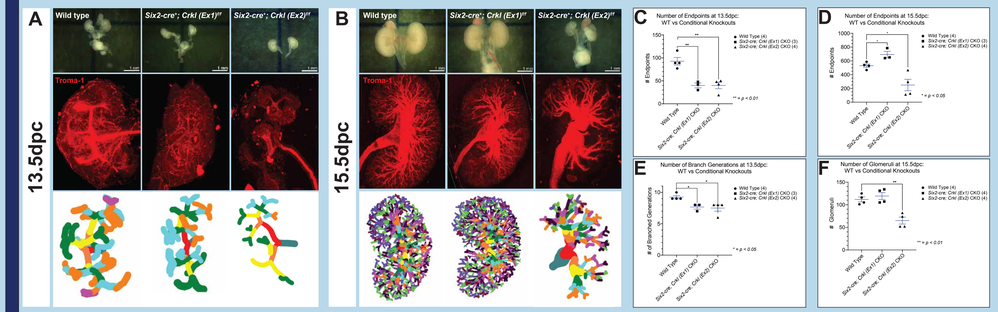
Selective and Conditional Ablation of Crkl Isoforms Recapitulates CAKUT Phenotypes of 22q11.2/DiGeorge Syndrome

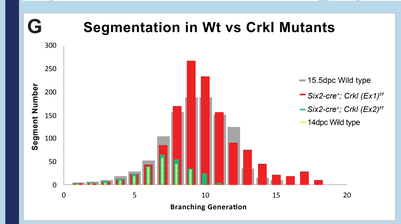


A-V: Dissection and H&E images taken from each cohort within the series of conditional deletions. **(A-C)** Removal of both *Crkl* splice variants from the MM yields severe phenotypes at the marco- and microscopic levels, including malformed glomeruli (C, yellow arrows), while removal of only T1 **(D-F)** also yields a severe phenotype, albeit with lower frequency. **(H-J)** Removal of both splice variants from both the MM and UB, or of only T1 **(K-M)** lead to upper and lower urinary tract defects, including hypoplasia, which was the most common phenotype in both groups. **(N-P)** Removal of both splice variants in the UB had no effect on phenotype, whereas removal of only T1 **(Q-S)** lead to dilations at the UPJ (R) in 30% of mice. **(T-V)** Age-matched wild-type. KA = Kidney anomaly, OU = Obstructive Uropathy. All images are from animals at 3 weeks gestation (18.5dpc - P0).

18.5 – P0	Six2-Cre;		Pax2-cre;		Hoxb7-cre;		Total Wild-types	
	(Ex1) f/f	(Ex2) f/f	(Ex1) f/f	(Ex2) f/f	(Ex1) f/f	(Ex2) f/f	Ex1*	Ex2*
KA	4 / 5 (80%)	4 / 8 (50%)	11 / 14 (79%)	7 / 9 (78%)	0 / 7	0 / 10	0 / 88	0 / 119
OU	0 / 5	0 / 8	5 / 14 (36%)	2 / 9 (22%)	0 / 7	3 / 10 (30%)	0 / 88	0 / 119

Loss of both Crkl isoforms leads to an overbranched phenotype, while genetic ablation of only T1 leads to developmental delay

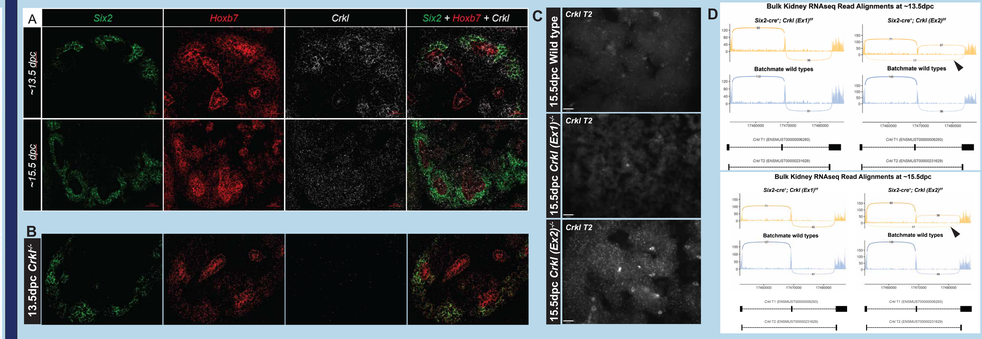




(A) 13.5dpc and **(B)** 15.5dpc embryonic kidneys across each genotype representing the diversity of branching phenotypes. 3D reconstructions of Troma1/Ck8-stained tissues (middle) were skeletonized and quantified. At 13.5dpc, endpoints, or tips **(C)**, are decreased in each genotype, however, at 15.5dpc **(D)** Ex1 mutants exhibit an increased # of tips, while Ex2 mutants remain underdeveloped. Relatedly, the early delay in both phenotypes is also represented in the number of branch generations at 13.5dpc **(E)**. By 15.5dpc, Ex1 mutants normalize their number of glomeruli, with Ex2 mutants continuing to lag **(F)**. **(G)** Lastly, an overlay of branch generations from representative samples across each genotype at 15.5dpc compared to data from 15.5dpc wild-type and historical datasets from 14.5dpc wild type mice highlight the developmental delay in Ex2 mutants .

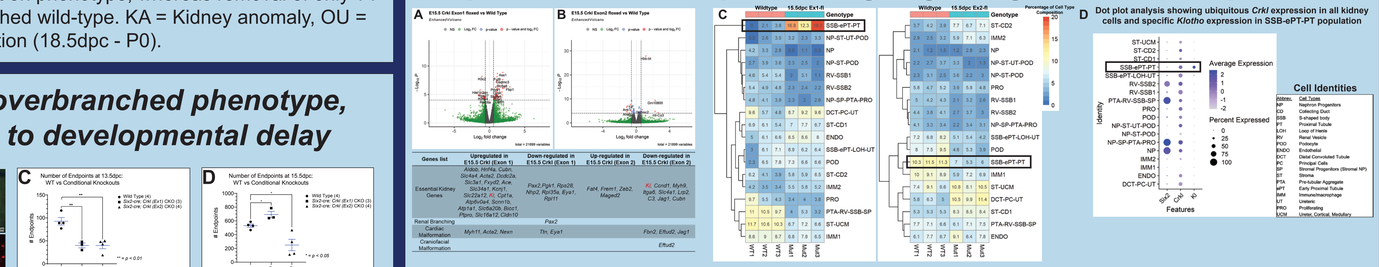
Results

Removal of Crkl exon 1 results in complete knockout, while selective ablation of exon 2 leads to a functional overexpression of Crkl T2



In a series of fluorescent RNAscope ISH assays, probes against *Six2*, *Hoxb7* and *Crkl* were tested on wild-type tissues at **(A)** 13.5 and 15.5dpc, and **(B)** in tissues from a global *Crkl* knockout. **(C)** A *Crkl T2*-specific BaseScope probe, *targeting the Ex1-Ex3 splice junction,* was used on tissues from 15.5dpc wild-type and global mutant mice, where there is an observed absence of T2 in *Crkl (Ex1)*$^{-/-}$ mutants, but an un-*expected increase* in the expression of T2 in *Crkl (Ex2)*$^{-/-}$ global mutants. **(D)** Sashimi plots of bulk RNAseq data show raw reads coverage within and across *Crkl* exons & junctions (resp.). Peaks indicate greater read coverage; curved lines represent coverage of exon-exon junctions. Note increased coverage of the Ex1-Ex3 splice junction (arrowheads) in *Six2-cre*$^{+}$*;Crkl (Ex2)*$^{f/f}$ mice, where only T2 can be expressed, indicating higher expression vs wild type or *Six2-cre*$^{+}$*;Crkl (Ex1)*$^{f/f}$ mutants.

Bulk RNA-seq coupled with supervised single cell deconvolution implicates Klotho-dependent Fgf signaling in SSB development



Differential expression analysis of bulk RNAseq data from **(A)** *Six2-cre*$^{+}$*;Crkl (Ex1)*$^{f/f}$, and **(B)** *Six2-cre*$^{+}$*;Crkl (Ex2)*$^{f/f}$ at 15.5dpc show more dis-regulated genes in the absence of T1 and T2 compared to the loss of only T1. **(C)** Deconvolution of bulk RNA-seq to pseudo scRNA-seq (Naganuma H., *et al.*, Dev Biol 2021 Feb;470:62-73) shows increase in genes expressed by the SSB-ePT-PT cell population in the *Six2-cre*$^{+}$*;Crkl (Ex1)*$^{f/f}$ mutant, and depletion of these genes in the *Six2-cre*$^{+}$*;Crkl (Ex2)*$^{f/f}$ mutant. **(D)** Klotho is uniquely expressed in this cell population and its mRNA is significantly and opposingly regulated across each genetic context

Conclusions & Future Directions

- This study confirms the causal role of *CRKL* in kidney malformations observed in individuals with the Chr. 22q11.2 microdeletion syndrome and demonstrates the requirement of both *Crkl* isoforms for normal urinary tract development.
- By selectively ablating one or both transcripts, data imply a negative and positive, resp., effect on regulation of Klotho-dependent proliferation of S-shaped bodies / early proximal tubule cells during early development.
- Based on the literature, it is likely that Fgf signaling (i.e. Fgf8 and Fgf10) is disregulated, starting from the *Six2*+ nephron progenitors where *Crkl* is genetically altered, providing a potential mechanism for the hypothesized Klotho-mediated branching phenotypes.
- Furthermore, Klotho-dependent Fgf signaling is likely to constitute a unifying cellular mechanism for the cardiac, craniofacial and urinary tract malformations related to genetic mutations in *CRKL*.

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