

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36

Tfap2a is a novel gatekeeper of differentiation in renal progenitors during kidney development

Brooke E. Chambers¹, Gary F. Gerlach¹, Karen H. Chen¹, Eleanor G. Clark¹, Ignaty Leshchiner²,
Wolfram Goessling², and Rebecca A. Wingert^{1*}

¹Department of Biological Sciences, Center for Stem Cells and Regenerative Medicine,
Center for Zebrafish Research, University of Notre Dame, Notre Dame, 46556, USA

² Brigham and Women's Hospital, Genetics and Gastroenterology Division,
Harvard Medical School, Harvard Stem Cell Institute, Boston, MA 02215, USA

*Corresponding author

Keywords: kidney, nephron, segmentation, differentiation, *tfap2a*, *tfap2b*, *irx3b*, *irx1a*, zebrafish

Key Abbreviations: branchio-oculo-facial syndrome (BOFS); corpuscle of Stannius (CS); distal early (DE); distal late (DL); fluorescent whole mount *in situ* hybridization (FISH); immunofluorescence (IF); *iroquois homeobox 1a* (*irx1a*); *iroquois homeobox 3b* (*irx3b*); hours post fertilization (hpf); mesenchymal to epithelial transition (MET); morpholino oligonucleotide (MO); proximal convoluted tubule (PCT); proximal straight tubule (PST); somite stage (ss); thick ascending limb (TAL); *transcription factor AP-2 alpha* (*tfap2a*); *transcription factor AP-2 beta* (*tfap2b*); whole mount *in situ* hybridization (WISH); wild-type (WT)

Correspondence: Rebecca A. Wingert, Ph.D., Department of Biological Sciences, University of Notre Dame, 100 Galvin Life Sciences, Notre Dame, IN 46556, USA; Email: rwingert@nd.edu, Phone: (574)-631-0907, Fax: (574)-631-7413

37 **Summary Statement**

38 Here, we report for the first time that *transcription factor AP-2 alpha (tfap2a)* controls the progression from
39 nephron progenitor into the fully differentiated state. This fundamentally deepens our knowledge about the
40 genetic control of kidney development.

41

42

43 **Abstract**

44 Renal functional units known as nephrons undergo patterning events during development that create a
45 segmental array of cellular populations with discrete physiological tasks. Knowledge about the terminal
46 differentiation programs of each nephron segment has central importance for understanding kidney
47 disease and to advance regenerative medicine, as mammalian nephrons grown in organoid cultures from
48 pluripotent cells fail to terminally differentiate. Here, from a novel forward genetic screen using zebrafish
49 we report the discovery that *transcription factor AP-2 alpha (tfap2a)* coordinates a gene regulatory network
50 that controls the progression of nephron distal segment progenitors into the differentiated state.
51 Overexpression of *tfap2a* rescued differentiation in mutants and caused ectopic expression of distal
52 segment markers in wild-type nephrons, indicating *tfap2a* is sufficient to instigate the distal segment
53 differentiation program. *tfap2a/2b* deficiency exacerbated distal nephron segment differentiation defects,
54 revealing functional redundancy where *tfap2a* has a dominant role upstream of its family member. With
55 further genetic studies, we assembled a blueprint of the *tfap2a* gene regulatory network during
56 nephrogenesis. We demonstrate that *tfap2a* acts downstream of *Iroquois homeobox 3b*, a conserved distal
57 lineage transcription factor. *tfap2a* controls a circuit consisting of *irx1a*, *tfap2b*, and genes encoding solute
58 transporters that dictate the specialized metabolic functions of the distal nephron segments, and we show
59 for the first time that this regulatory node is distinct from the pathway circuits controlling aspects such as
60 apical-basal polarity and ciliogenesis during the differentiation process. Thus, our studies reveal new
61 insights into the genetic control of differentiation, where *tfap2a* regulates the suite of segment transporter
62 traits. These findings have relevance for understanding renal birth defects, as well as efforts to recapitulate
63 nephrogenesis *in vivo* to make functional units that can facilitate organoid applications such as drug
64 discovery and regenerative therapies.

65 Introduction

66 Vertebrate kidney ontogeny involves the reiterative formation and degradation of several structures from
67 the intermediate mesoderm: the pronephros, the mesonephros, and the metanephros (Saxen, 1987). In
68 amniotes, the metanephros serves as the final kidney form, while in lower vertebrates, such as fish and
69 frogs, the mesonephros functions as the adult kidney. Importantly, all the kidney versions are comprised of
70 conserved functional units called nephrons (Dressler, 2006). The nephron is comprised of a blood filter, a
71 segmented epithelial tubule, and a collecting duct. Each of these anatomical nephron parts modifies the
72 filtrate in a stepwise fashion to perform the vital tasks of excretion, pH balance, and fluid homeostasis.
73 Occurring in approximately 1 in 500 births, Congenital Anomalies of the Kidney and Urinary Tract (CAKUT)
74 are among the most common birth defects and are the primary cause of pediatric end stage renal disease
75 (ESRD) (Airik and Kispert, 2007; Song and Yosypiv, 2011). The shared etiology across these diverse
76 conditions is the aberrant development of nephrons stemming from genetic dysregulation (Schedl, 2007).
77 To this end, it is imperative to understand the signals that coordinate nephron formation during renal
78 organogenesis.

79
80 The zebrafish pronephros has emerged as a genetically tractable vertebrate model to study the molecular
81 mechanisms regulating nephron segment development events (Wingert et al., 2007; Wingert and
82 Davidson, 2008; Wingert and Davidson, 2011). The embryonic zebrafish is transparent in nature, and its
83 pronephric kidney is structurally simple, consisting of two bilateral nephrons that make it an excellent
84 model to study renal progenitor changes *in vivo* (Naylor et al., 2017). Like other vertebrate nephrons, the
85 zebrafish pronephros is patterned into distinct proximal and distal epithelial segments (Wingert et al., 2007;
86 Wingert and Davidson, 2008). Further, zebrafish mirror fundamental processes of mammalian nephron
87 formation such as the mesenchymal to epithelial transition (MET) of renal progenitors, establishment of
88 apical-basal polarity, lumen formation, ciliogenesis, and formation of specialized segment populations
89 (Gerlach and Wingert, 2013). While there has been significant progress in understanding nephron segment
90 patterning in recent years (Desgrange and Cereghini, 2015; Lindström et al., 2015; Chung et al., 2017), the
91 pathways that dictate segmental terminal differentiation are far from understood.

92
93 Transcription factors play a central role in operating the genetic networks that orchestrate renal cell fate
94 acquisition and nephron segment patterning (Desgrange and Cereghini, 2015; Lindström et al., 2015).
95 Advances in single-cell RNA sequencing and gene expression analysis in both the embryonic murine and
96 human kidneys have brought to light an inventory of factors mapped to distinct regions of developing
97 nephrons (Lindström et al., 2018a,b). Although these studies have provided a detailed transcription factor
98 localization atlas that is time-dependent, the functions of these factors in nephrogenesis have not yet been
99 fully elucidated. One uncharacterized gene is *Transcription Factor AP-2 Alpha (TFAP2A)*, which clustered
100 with developing medial/distal tubule signatures (Lindström et al., 2018b). *TFAP2A* is a member of the AP-2
101 transcription factor family (AP-2 α , AP-2 β , AP-2 γ , AP-2 δ and AP-2 ϵ), whose proteins share highly

102 conserved dimerization and DNA binding motifs across vertebrates (Fig. S1). AP-2 factors bind to GC-rich
103 promoter sequences, and can homodimerize and heterodimerize with one another (Eckert et al., 2005).
104 During development, these factors have been shown to exercise redundant and unique functions
105 depending on the tissue context (Eckert et al., 2005).

106
107 *Tfap2a* and family member *Tfap2b* also have overlapping expression patterns during vertebrate
108 embryogenesis in neural crest derivatives, surface ectoderm, and the kidney (Moser et al., 1997; Knight et
109 al., 2003; Knight et al., 2005). Surprisingly, the elimination of *Tfap2a* and *Tfap2b* in mice results in
110 completely different phenotypic outcomes. *Tfap2a* knockout mice die perinatally and display a suite of
111 pleiotropic features that include craniofacial alterations, incomplete neural tube closure, hypoplastic hearts
112 and kidneys (Schorle et al., 1996; Zhang et al., 1996; Brewer and Williams, 2004; Brewer et al., 2004). In
113 contrast, *Tfap2b* null mice exhibit patent ductus arteriosus and die shortly after birth due to acute renal
114 failure with elevated apoptosis (Moser et al., 1997; Hilger-Eversheim et al., 2000; Wang et al., 2018).
115 Because *Tfap2b* mutants exhibit less severe phenotypes, this factor is proposed to share redundant
116 functions with *Tfap2a* during development (Eckert et al., 2005; Kerber et al., 2001). An example in support
117 of this relationship is that *Tfap2a* plays a more dominant role than *Tfap2b* in the development of branchial
118 arches in mice (Van Otterloo et al., 2018).

119
120 Genetic defects in the AP-2 factors are associated with several human diseases. Autosomal dominant
121 *TFAP2A* mutations in humans cause branchio-oculo-facial syndrome (BOFS), which primarily affects
122 craniofacial tissue (Milunsky et al., 2008). Additionally, human *TFAP2A* lesions are associated with
123 multicystic dysplastic kidney defects, but the mechanisms have remained unexplored. Dominant-negative
124 mutations in human *TFAP2B* cause Char Syndrome, which affects heart, face, and limb development
125 (Satoda et al., 2000). Despite the previously documented renal phenotypes associated with *Tfap2a* and
126 *Tfap2b* deficiency in rodents, these factors have not been studied further in the context of kidney
127 development. For example, nephron segmentation has not been analyzed in either *Tfap2a* or *Tfap2b*-
128 deficient murine models. Nevertheless, *Tfap2a/tfap2a* has been extensively studied in the vertebrate
129 neural crest, where it facilitates specification and differentiation through a complex genetic regulatory
130 network (Knight et al., 2003; Knight et al., 2005; Holzschuh et al., 2003; Barrallo-Gimeno et al., 2003;
131 O'Brien et al., 2004; Li and Cornell, 2007; Hoffman et al., 2007; Van Otterloo et al., 2010; de Croz e et al.,
132 2011; Wang et al., 2011; Bhat et al., 2012; Green et al., 2014; Kantarci et al., 2015; Seberg et al., 2017).
133 These studies provide a valuable framework with which to consider the roles of *Tfap2a/tfap2a* in other
134 tissues, where it is likely to also mediate genetic networks.

135
136 Here, we report the novel zebrafish nephron segment mutant, *terminus (trm)*, which was isolated in a
137 forward haploid genetic screen. Employing whole genome sequencing, we identified a mutation that blocks
138 proper splicing of *tfap2a*. While *tfap2a* deficient nephrons have normal distal segment pattern formation,

139 display normal epithelial polarity and cilia development, they experience a block in other aspects of
140 terminal differentiation, resulting in the loss of solute transporter expression within distal segments.
141 Interestingly, *tfap2a* is sufficient to induce ectopic expression of distal segment markers in adjacent
142 segment domains. We found that *tfap2b* functions redundantly and downstream of *tfap2a* to turn on the
143 distal nephron solute transporter program. Further, *tfap2a* articulates with the Iroquois homeobox
144 transcription factors *irx1a* and *irx3b*, which are regulators of intermediate/distal nephron identity. Our study
145 reveals for the first time that *tfap2a* controls a gene regulatory network that serves as a gatekeeper of
146 terminal differentiation during nephron segment development, and establishes a new paradigm that will be
147 valuable to deepen our knowledge of cell differentiation mechanisms in the kidney.

148

149

150

151

152

153 **Results**

154 **Forward genetic screen identifies *tfap2a* as a novel regulator of nephron development**

155 There remain many gaps in our understanding of the genetic blueprint needed to orchestrate renal stem
156 cell fate decisions and nephron segment formation during kidney ontogeny. The embryonic zebrafish
157 kidney, or pronephros, is a practical genetic model for nephron segmentation (Gerlach and Wingert, 2013).
158 At 24 hours post fertilization (hpf), the pronephros is fully formed and exhibits a very simple organization
159 consisting of two parallel nephrons (Fig. 1A), making cellular changes easy to detect (Poureetezadi and
160 Wingert, 2016). Each nephron is comprised of a blood filter, a series of proximal and distal segments that
161 reabsorb and secrete molecules, and a collecting duct to transport waste (Fig. 1A) (Wingert et al., 2007).

162

163 To identify novel renal regulators, we performed a forward genetic haploid screen in zebrafish. We
164 obtained maternal gametes from the F1 generation, applied ultraviolet light inactivated sperm to generate
165 F2 haploid embryos, and assayed them for nephron segment defects by whole mount *in situ* hybridization
166 (WISH) (Kroeger et al., 2014). For our assay we applied a mixture of probes that specifically localize to
167 alternating compartments of the pronephros: podocytes (*wt1b*), proximal convoluted tubule (PCT)
168 (*slc20a1a*), and the distal early segment (DE) (*slc12a1*) (Fig. 1B). Through this multiplex assay we isolated
169 the nephron mutant *terminus* (*trm*) which affected DE segment development based on abrogated *slc12a1*
170 expression within the pronephros (Fig. 1B).

171

172 We performed whole genome sequencing to identify the causative gene for the *trm* phenotype (Leshchiner
173 et al., 2012). Using SNP track software analysis, the location of the genetic lesion was mapped to
174 chromosome 24 (Fig. 1C). We used previously described thresholds to enrich results (Ryan et al., 2013)
175 and discovered the gene *tfap2a* was a high scoring candidate at the chromosome 24 locus, where there

176 was a G → A substitution that was predicted to disrupt splicing at the splice donor site of exon 1. We
177 performed direct PCR sequencing on *trm* mutants and wild-type (WT) siblings, and confirmed this genetic
178 change (Fig. 1C). To characterize how this mutation affected splicing, we conducted transcript analysis.
179 RT-PCR on total RNA isolated from *trm* mutants revealed four aberrant *tfap2a* spliceoforms compared to
180 WTs (Fig. 1D). One aberrant transcript encoded an in-frame addition of 38 amino acids (Fig. 1D), which
181 may possess native function or have dysfunctions associated with protein folding or stability. The other
182 three transcripts encoded premature stop codons (Fig. 1D). These aberrant *trm* transcripts are predicted to
183 truncate the essential transcriptional activation and DNA binding domains in the Tfap2a protein (Fig. 1D).

184

185 Next, we explored whether the loss of *tfap2a* function in *trm* mutants was the sole origin of their renal
186 phenotype. To do this, we performed complementation tests between *trm* and *tfap2a*^{m819}, the latter which
187 encodes a nonsense allele, followed by phenotype assessment with WISH and finally genotyping analysis.
188 Compound *trm*^{+/-};*tfap2a*^{m819+/-} heterozygote embryos displayed the loss of *romk2* expression within the
189 pronephros DE segment and reduced *dlx2* expression in the neural crest as well (Fig. 1E). This result
190 indicates that the alleles failed to complement one another, which most likely indicates that the same gene
191 is affected, and provided powerful evidence that disruption of *tfap2a* expression alone underlies the *trm*
192 phenotype.

193

194 Until now, *tfap2a* has been known as essential for neural crest and epidermis differentiation. Thus, we
195 assessed whether *trm* mutants evinced hallmarks of *tfap2a* deficiency. With analysis of live morphology at
196 4 dpf, we found that *trm* developed abnormal craniofacial cartilage and pericardial edema, which was
197 phenocopied upon *tfap2a* morpholino knockdown (Fig. 1F). RT-PCR analysis confirmed that this *tfap2a*
198 MO effectively disrupts splicing (Fig. S2). We examined facial cartilage using Alcian blue staining, where
199 we found *trm* possesses defects in Meckel's cartilage and pharyngeal arch structures (Fig. 1G). The
200 cartilage phenotypes observed in *trm* are consistent with the documented neural crest *tfap2a* mutant
201 alleles *lockjaw* and *mont blanc* (Knight et al., 2003; Barrallo-Gimeno et al., 2003). *trm* also displayed
202 disrupted craniofacial vasculature formation as indicated by o-dianisidine staining (Fig. S4). Further, when
203 we assayed for *tfap2a* protein by whole mount immunofluorescence (IF), we detected no pronephric
204 expression in *trm* mutants as compared to WT at 24 hpf. The absence of *tfap2a* protein expression in the
205 mutant pronephros indicates that the ultimate consequence of *trm* allele is a bona-fide loss-of-function. In
206 light of the mutation site, these IF data further indicate that the *tfap2a* exon1c spliceoform encodes the
207 dominant protein variant that is active during kidney development. In sum, these results show that *trm*
208 mutants exhibit many features of *tfap2a* deficiency, and reveal for the first time that *tfap2a* is needed for
209 nephrogenesis—specifically for proper emergence of the DE segment population.

210

211

212 ***tfap2a* and *tfap2b* are coexpressed dynamically during nephron development and function**
213 **redundantly to induce regimens of distal segment solute transporter genes**

214 Of the AP-2 family of transcription factors, only *tfap2a* and *tfap2b* have been reported as expressed in the
215 developing zebrafish pronephros (Knight et al., 2005; Sugano et al., 2017). Zebrafish *tfap2a* and *tfap2b*
216 genes are closely related, as they share overall 65% amino acid sequence identity, with highly similar
217 DNA-binding and transactivation domains in particular (Knight et al., 2005). The sequence of both *tfap2a*
218 and *tfap2b* zebrafish genes are conserved with their respective vertebrate orthologues as well (Fig. S1)
219 (Knight et al., 2005).

220
221 To further investigate the spatiotemporal expression domains of *tfap2a* and *tfap2b* within the developing
222 renal field, we performed WISH with RNA antisense riboprobes over the time span of nephrogenesis. We
223 found that *tfap2a* and *tfap2b* transcripts were expressed broadly in renal progenitors at the 10 somite stage
224 (ss), however this expression became dynamically restricted to the distal region of the pronephros by the
225 28 ss (Fig. 2A). Through fluorescent *in situ* hybridization (FISH) studies, we confirmed that *tfap2a* and
226 *tfap2b* were robustly co-expressed in nearly identical renal progenitor domains at the 10 ss (Fig. 2B). FISH
227 at the 20 ss and 28 ss revealed that *tfap2a* had a mostly broader expression pattern across the distal
228 pronephros compared to *tfap2b* (Fig. 2B), consistent with expression in the DE and DL segments.
229 Differential *tfap2a/b* expression was noted rostrally and in the posterior pronephric duct region at the 20 ss
230 and 28 ss, where only *tfap2a* transcripts were detected (Fig. 2B). Next, we sought to validate if *tfap2a* was
231 expressed in the DE segment domain, which is demarcated by *slc12a1* expression, given the *trm* mutant
232 phenotype (Wingert et al., 2007). *tfap2a* and *slc12a1* transcripts were co-localized at the 28 ss (Fig. 2C).
233 These results indicate that *tfap2a* and *tfap2b* expression occurs in renal progenitors during a
234 developmental window that positions them as possible participants in distal nephron development.

235
236 To explore potential genetic relationships between *tfap2a* and *tfap2b*, we performed loss of function
237 experiments. *trm* exhibited significantly reduced *tfap2b* expression in the distal pronephros and the
238 hindbrain region (Fig. 2D). Conversely, knockdown of *tfap2b* using a morpholino (MO) strategy revealed
239 that *tfap2a* expression was unaffected throughout the embryo, including the pronephros (Fig. 2E). The
240 *tfap2b* MO tool was verified to interrupt splicing by RT-PCR, which revealed that it caused inclusion of
241 intronic sequence that encoded a premature stop codon, which is predicted to generate a truncated
242 peptide (Fig. S3). In combination, these genetic studies suggest a more dominant role of *tfap2a* in the
243 context of nephrogenesis, placing *tfap2a* upstream of *tfap2b*.

244
245 Because *tfap2a* and *tfap2b* have been demonstrated to function redundantly in the development of other
246 tissue types, we next wanted to determine if these two factors could act similarly during nephrogenesis
247 (Knight et al., 2005; Van Otterloo et al., 2018; Seberg et al., 2017; Bassett et al., 2012; Jin et al., 2015). To
248 interrogate this, we performed combination knockdown studies and assayed a set of solute transporters

249 that characterize the distal nephron segments at 24 hpf (Fig 3A). *tfap2b* deficiency alone had no detectable
250 affects on distal solute transporter expression. *trm* and *tfap2a* morphants exhibited significant reductions in
251 *slc12a1*, *slc12a3*, and *clcnk* expression as compared to WT (Fig. 3A,B,C,D). At 4 dpf, *trm* mutants still
252 failed to express distal early solute transporters *slc12a1* and *romk2* (Fig. S4). However, the pronephros
253 was functional between 2 and 3 dpf, based on assessment of renal clearance, which normally initiates
254 during this time period, thereby ruling out developmental delay (Fig. S4). Knockdown of *tfap2b* in *trm*
255 mutants caused a more severe *slc12a3* reduction than *tfap2a* deficient embryos (Fig. 3). Interestingly,
256 there was not a statistically significant reduction in the *slc12a1* or *clcnk* domain length in *tfap2b* injected
257 *trm* mutants versus *tfap2a* deficiency alone (Fig. 3). By comparison, *tfap2a/2b* morphants had statistically
258 significant reduction of the *slc12a1*, *slc12a3*, and *clcnk* pronephros expression domains versus *tfap2a*
259 deficiency alone. Notably, *tfap2a* morpholino targets all three splice variants, however the *trm* mutation
260 only affects one of these splice variants (Fig. 1C). In light of this phenotypic spectrum, we concluded that
261 the development of the distal nephron program is sensitive to the dosage of functional *tfap2a/2b* alleles
262 that are present. Taken together, these genetic studies reveal that the concerted action of *tfap2a* and
263 *tfap2b* is necessary to fully turn on distal solute transporter programs, where *tfap2a* plays a more
264 prominent role in this process upstream of *tfap2b*.

265

266

267 ***tfap2a* is necessary and sufficient for DE differentiated cell expression signature**

268 Next, we wanted to determine if provision of WT *tfap2a* transcripts could specifically rescue the absence of
269 DE solute transporter expression in *trm*. Activation of a heat-shock inducible *tfap2a* transgene at the 8 ss
270 restored *romk2* expression in *trm* mutants comparable to WT levels based on absolute length
271 measurements (Fig. 4A). This result further underscores the conclusion that *tfap2a* deficiency is the single,
272 specific cause of the *trm* phenotype. We then performed *tfap2a* gain of function studies by two
273 independent methods: 1) employing an inducible *hs:tfap2a* transgenic line and 2) microinjection of *tfap2a*
274 mRNA in WT embryos. When we overexpressed *tfap2a* by these approaches there was a significant
275 expansion of the *romk2* expression domain which normally marks the DE segment (Fig. 4B). All of the
276 heat-shock treated *hs:tfap2a* transgenic embryos exhibited an expanded *romk2* domain, while control non-
277 heat-shocked embryos developed normal *romk2* domains. About 9% (12/132) of the *tfap2a* cRNA injected
278 clutches presented with an increased *romk2* domain, however about 64% (85/132) of embryos were
279 scored as dysmorphic. This lower phenotype penetrance, compared to the transgenic overexpression
280 model, is likely caused by the toxic affects of *tfap2a* during early development, which has been reported to
281 disrupt gastrulation (Li and Cornell, 2007). Interestingly, ectopic *romk2*⁺ cells appeared to invade both the
282 adjacent proximal and distal segment domains in these gain-of-function experiments.

283

284 To examine more closely if *tfap2a* overexpression was inducing neighboring nephron segments to convert
285 to a DE program, we performed double FISH on heat-shocked *hs:tfap2a* embryos. We detected *slc12a1*⁺

286 cells within the *slc12a3*⁺ DL domain (Fig. 4C). Upon closer analysis, these cells were found to coexpress
287 both *slc12a1* and *slc12a3* transcripts (Fig. 4C'). Heat-shocked *hs:tfap2a* animals also possessed *slc12a1*⁺
288 cells spanning across the proximal domain that were coexpressing *slc9a3* (Fig. 4D,D'). These phenotypes
289 greatly contrast the WT situation, where there are sharp, clear boundaries between neighboring segment
290 domains in the nephron (Fig. 4C,D). These results indicate that *tfap2a* overexpression is sufficient to sway
291 the differentiation profile of proximal and distal late cell types by triggering the misexpression of DE-
292 specific solute transporters.

293

294

295 ***tfap2a* drives DE terminal differentiation program**

296 Previous studies have demonstrated *tfap2a* can regulate terminal differentiation of various cell types
297 including migratory neural crest, melanocytes, statoacoustic ganglion neurons, noradrenergic neurons, and
298 the trophoblast lineage (Barrallo-Gimeno et al., 2003; Kantarci et al., 2015; Seberg et al., 2017; Greco et
299 al., 1995; Kim et al., 2001; Pfisterer et al., 2001; Handwerger, 2009). This literature, in light of our loss of
300 function and gain of function results, led us to hypothesize that *tfap2a* controls the terminal differentiation
301 of distal nephron cells. To explore this notion, we first wanted to determine if the nephron segments were
302 patterned correctly in *trm* mutants.

303

304 To assess the pattern formation of nephron segments in *trm* mutants, we performed double WISH to
305 assess the segment domains located adjacent to the DE, in this case the pan-proximal and DL. *trm*
306 mutants exhibited a domain of *slc9a3* expression comparable to WT embryos (Fig. 5A). In both WT and
307 *trm*, this *slc9a3*⁺ region was followed by a gap situated at the position normally occupied by the DE
308 segment, and the *slc12a3* expression domain, which is smaller in *trm* mutants, immediately followed this
309 gap (Fig. 5A). The intact sequence of the pan-proximal, gap/placeholder, and then the DL segment
310 suggested that the DE segment 'footprint' was present in *trm* mutants, and thus that pattern formation had
311 proceeded during nephrogenesis (Fig. 5A). Additionally, *trm* mutants exhibited no alterations in *slc20a1a*
312 or *trpm7* expression domains, which mark the PCT and PST segments, respectively (Fig. S5). *tfap2a*
313 morphants also developed normal proximal segments, as well as the DE footprint (data not shown). These
314 results indicate that *tfap2a* deficient embryos undergo normal segmental patterning of the nephron tubule.

315

316 We then examined development of the corpuscle of Stannius in *trm* mutants, which is an endocrine gland
317 situated between the DE and DL (Cheng and Wingert, 2015). We used WISH to assess the expression of
318 transcripts encoding *stanniocalcin 1* (*stc1*), a specific CS marker. Compared to WT embryos, *trm* mutants
319 exhibited severely reduced *stc1* expression (Fig. S5). In sum, these data rule out the occurrence of
320 possible fate switches with adjacent nephron cell types that could account for the loss of the DE marker
321 expression, and suggest that in addition to the DE, *tfap2a* may regulate CS lineage development and/or
322 CS differentiation.

323

324 Next, we wanted to determine if *trm* mutant cells occupying the DE region were specified as kidney. To do
325 this, we individually assayed for the expression of genes that are expressed robustly throughout the entire
326 nephron tubule. Identical to WT embryos, *trm* mutants showed no gaps in expression of *cdh17* and *hnf1ba*,
327 illustrating that mutant DE cells were fated to a kidney lineage identity (Fig. 5B). Further, we assessed if
328 any alterations in cell proliferation or cell death occurs in response to loss of *tfap2a*. We detected no visible
329 changes in the number of pH3⁺ cells within the DE domain of *trm* mutants compared to WT controls at 24
330 hpf (Fig. 5C). Additionally, there were no perceivable differences in the number of dying cells labeled with
331 acridine orange in the distal pronephros compared to WTs at 24 hpf (Fig. 5D).

332

333 Renal progenitor differentiation in the zebrafish pronephros is known to entail an MET of the mesenchymal
334 progenitors, with establishment of polarity and lumen formation, as well as changes in cellular organelles
335 such as cilia. Therefore, we sought to determine whether *trm* mutant DE cells exhibited any of these
336 various differentiated features of the nephron tubular epithelium. Differentiated pronephros cells exhibit
337 apical-basal polarity and form either a primary cilium or multiple cilia by the 24 hpf stage (Gerlach and
338 Wingert, 2013, 2014; McKee et al., 2014; Marra and Wingert 2016; Marra et al., 2016). We performed
339 whole mount IF to analyze the expression of basolateral marker Na,K-ATPase and the apical adaptor
340 complex aPKC in the distal early region at 4 dpf. We found Na,K-ATPase and aPKC proteins were
341 properly localized in *trm* mutants as compared to WT, therefore indicating that epithelial polarity was
342 correctly established within the nephron tubules (Fig. 5E,E'). Further, *trm* mutants had a clearly discernible
343 tubule lumen, indicating that tubulogenesis had proceeded analogous to WT embryos (Fig. 5E,E'). Next,
344 we combined FISH of *slc12a1* with whole mount IF of acetylated tubulin to determine if cilia formation
345 occurs within the mutant DE segment region. At 24 hpf, cilia arrangement and morphology in *trm* mutants
346 was comparable to WT (Fig. 5F). This indicates that cilia assembly occurred normally in the mutant DE
347 cells, which were visualized based on their nearly abrogated *slc12a1* signal (Fig. 5F). Taken together,
348 these data indicate that mutant DE cells exhibit mature epithelial qualities, however cannot fully turn on
349 specific solute transporters, which are indicators of terminal differentiation and ultimately dictate segment-
350 specific physiological functions. From this, we conclude that *trm* mutants exhibit a unique block in the
351 terminal differentiation of distal nephron cells, which involves the acquisition of segment-specific solute
352 transporter proteins but is not linked to MET, polarity establishment, tubulogenesis or ciliogenesis
353 programs.

354

355

356 ***tfap2a* functions downstream of *irx3b* and upstream of *irx1a* in the distal pronephros**

357 We next wanted to understand the genetic relationship of *tfap2a* with known segment patterning factors, to
358 establish the hierarchical regulation of *tfap2a* during distal segment development. Previous studies have
359 shown *Ir3/irx3b* are required for the development of *slc12a1*⁺ distal tubule cells in *Xenopus* and zebrafish,

360 respectively (Wingert and Davidson, 2008; Reggiani et al., 2007; Marra and Wingert, 2014). Because of
361 this requirement, we first selected *irx3b* as a putative *tfap2a* gene regulatory network candidate for
362 investigation.

363

364 To determine if *tfap2a* and *irx3b* are co-expressed during pronephros development, we performed double
365 FISH studies. We found *tfap2a* and *irx3b* transcripts were co-localized in developing distal nephron cells at
366 the 20 ss (Fig. 6A). Because of the *tfap2a/irx3b* overlapping expression patterns, we rationalized that these
367 factors could be interacting in the same developmental pathway. Therefore, we performed knockdown
368 experiments to determine potential pathway interactions between *tfap2a* and *irx3b*. Upon *tfap2a*
369 knockdown, the *irx3b* expression domain was unchanged (Fig. 6B). However, upon *irx3b* knockdown, the
370 *tfap2a* expression domain was significantly truncated (Fig. 6B,C). The regional loss of *tfap2a* transcripts in
371 *irx3b* morphants equates to the DE segment address. We also observed a loss of *tfap2a* expression in
372 migrating neural crest streams in *irx3b* morphants (Fig. 6B). To further validate if *tfap2a* acts downstream
373 of *irx3b*, we performed rescue experiments in *irx3b* morphants. Overexpression of the *hs:tfap2a* transgene
374 was unable to rescue *romk2* expression in *irx3b* knockdowns (data not shown). We postulate this is
375 because *irx3b*-deficiency causes loss of *hnf1ba* expression within the DE progenitor compartment,
376 therefore these cells are not competent to respond to Tfp2a activity (Naylor et al., 2013). These results
377 suggest that *tfap2a* activates the DE program downstream of *irx3b*.

378

379 Previous literature has determined *Irx1* and *Irx3* are dually required for *Xenopus* pronephros development
380 (Reggiani et al., 2007; Alarcon et al., 2008). Importantly, loss of *Irx1* in *Xenopus* results in proximal
381 downregulation of the *Nkcc2* expression domain, the *slc12a1* equivalent in zebrafish (Reggiani et al.,
382 2007). Therefore, we chose Iroquois homeobox family member, *irx1a*, as another important molecular
383 player for analysis. In WT embryos, *irx1a* transcript expression was primarily localized to the DE segment
384 (Fig. 6D) (Cheng et al., 2001). In *trm* mutants and *tfap2a* morphants, *irx1a* expression was nearly
385 abrogated, with only a few remaining nephron cells expressing transcripts (Fig. 6D). When we induced
386 overexpression of *tfap2a* at the 8 ss, the *irx1a* expression domain length was significantly expanded,
387 indicating that *tfap2a* functions to activate *irx1a* expression directly or indirectly (Fig. 6E,F). Our results
388 place *tfap2a* function with respect to *irx* activity, indicating that *tfap2a* functions downstream of *irx3b* and
389 upstream of *irx1a* during distal segment differentiation. Taken together, our genetic analyses suggest a
390 working model in which *tfap2a* coordinates a genetic regulatory network, likely through direct and indirect
391 interactions, that controls the differentiation of distal nephron progenitors (Fig. 7).

392

393

394

395

396

397 Discussion

398 Here, we have shown that *tfap2a* is required for distal nephron segment differentiation. We propose a
399 model in which *trm* mutant cells progress through normal nephron developmental checkpoints until the
400 final stage of differentiation (Fig. 7A). Our data supports the conclusion that *tfap2a* deficiency does not
401 affect the derivation of the kidney lineage from intermediate mesoderm, as the expression of *hnf1ba* and
402 *cdh17* are unaffected. Further, we found that *trm* undergo nephron specification and epithelialization, as
403 *tfap2a*-deficient cells exhibit segmental patterning, proper localization of polarity proteins, and form cilia.
404 However, mutant cells appear to be frozen nonetheless in a specified renal precursor state, as they fail to
405 turn on the suite of terminal differentiation genes that encode the distal solute transporters *slc12a1*, *romk2*,
406 and *clcnk*. Taken together, we conclude that *tfap2a* is required for a discrete genetic circuit during terminal
407 differentiation of the distal nephron epithelium.

408
409 Interestingly, this discovery disentangles the control of the solute transporter transcriptome from other
410 differentiation processes, such as MET and polarity establishment in renal progenitors. Additionally, we
411 have assembled a proposed Tfab2 genetic circuit that functions to achieve differentiation of distal nephron
412 epithelium within the zebrafish embryonic kidney (Fig. 7B). In this network, *tfap2a* functions upstream of
413 *tfap2b* as indicated by our genetic analyses. However, both *tfap2a* and *tfap2b* function synergistically in
414 renal progenitors to turn on distal solute transporter genes, a level of redundancy that likely serves to
415 amplify and reinforce this specific differentiation signal. This model is supported by the findings that
416 elimination of *tfap2a* alleles led to defects in solute transporter expression, however elimination of *tfap2b*
417 alleles alone had no consequence. Compound knockdown of *tfap2a* and *tfap2b* yielded the most severe
418 phenotype, unveiling this layer of functional redundancy. As the *trm* mutation affects only one of the *tfap2a*
419 spliceforms, it is interesting that disrupting only this transcript results in a kidney phenotype, and suggests
420 it encodes the sole protein variant that is fundamental for kidney development. The current study does not
421 reconcile whether *tfap2a* and *tfap2b* are interacting by direct or indirect modes of regulation, and whether
422 the suite of targets are direct or indirect (Fig. 7B). For example, it is possible *tfap2a* binds to the *tfap2b*
423 promoter region functioning as a transcriptional activator. It is also feasible Tfab2a heterodimerizes with
424 Tfab2b affecting the transcription of downstream target genes accordingly. These potential biochemical
425 mechanisms are crucial areas for future investigation.

426
427 Further, our genetic model supports the conclusion that *tfap2a* acts in the same pathway as Iroquois
428 homeobox genes *irx3b* and *irx1a*. Our genetic experiments indicate *irx3b* promotes *tfap2a* expression, and
429 *tfap2a* functions upstream of *irx1a* (Fig. 7). These genes have been previously implicated as necessary for
430 intermediate-distal early nephron development in zebrafish and frogs (Wingert and Davidson, 2011;
431 Reggiani et al., 2007). Importantly, Iroquois factors likely play conserved roles in mammalian counterparts,
432 as *Irx3* and *Irx1* define intermediate segment territories in developing S-shaped bodies (Reggiani et al.,

433 2007). Defining whether *Ir3b* directly regulates *Tfap2a*, and if *Tfap2a* directly regulates *irx1a*, will also be
434 important to discern in future studies as well.

435

436 Additionally, we discovered an intriguing phenotype when we globally overexpressed *Tfap2a*, where
437 *slc9a3*⁺ proximal tubule and *slc12a3*⁺ distal late tubule cells ectopically coexpress *slc12a1*, a marker of the
438 DE tubule program (Fig. 5). This indicates that renal progenitors are competent to respond to *Tfap2a*,
439 which is sufficient to activate the DE differentiation circuit. These mixed segment identities induced by
440 *Tfap2a* gain of function parallels a phenotype recently described as 'lineage infidelity,' which was observed
441 in differentiating nephrons of *Hox9,10,11*-knockout mice (Magella et al., 2018; Drake et al., 2018). These
442 studies similarly found individual cells that were dually expressing markers of more than one nephron
443 segment. Specifically, in E18.5 *Hoxa9,10,11/Hoxd9,10,11*-deficient kidneys, cells were scattered
444 throughout *Hnf4a*⁺ proximal tubules co-expressing collecting duct markers *DBA* and *Krt8*. Strikingly, mutant
445 kidneys sometimes possessed entire *Slc12a1*⁺ ascending loop of Henle (the DE analog in zebrafish)
446 domains that co-labeled with *Krt8*. These developing mutant nephrons undergo a normal segment
447 specification phase (E15.5), but later fail to maintain appropriate differentiation programs. It is an
448 interesting prospect that *Hox* mutants exhibit a normal specification phase, as our data similarly suggests
449 that *Tfap2a* is dispensable for nephron patterning, but necessary for inducing a proper differentiation state.
450 During zebrafish neural crest development, *Tfap2a* promotes expression of *Hox* group 2 genes to form
451 segments of the pharyngeal skeleton (Knight et al., 2003; Barrallo-Gimeno et al., 2003). Collectively, these
452 studies warrant *Hox* genes as important future areas of study regarding the *Tfap2* genetic regulatory
453 network controlling nephron differentiation.

454

455 With the advent of next-generation sequencing technologies, recent work in the field has identified new
456 targets within the *Tfap2* genetic regulatory network (Seberg et al., 2017). To do this, microarray analyses
457 and ChIP-seq were employed in tandem to identify novel players regulating melanocyte development
458 within the *Tfap2a* GRN. To collect candidates, microarray analysis of *Tfap2a*^{-/-} zebrafish trunks and *Tfap2a*-
459 deficient mouse melanocyte lines were conducted. Microarray results were compared to ChIP-Seq data
460 gathered from mouse and human melanocytes to determine which differentially regulated genes were
461 direct transcriptional targets of *Tfap2a*. The findings suggested *Tfap2a* directly regulates effectors of
462 melanocyte terminal differentiation (e.g. *dct*, *mlpha*, *mc2r*, *sox10*, *mitf*). Conducting microarray analysis of
463 our *trm* mutant zebrafish embryos and overlapping this data with the previously published mammalian
464 kidney *Tfap2a* Chip-Seq data set (Pihlajamaa et al., 2014) will help to identify putative direct targets
465 governing terminal differentiation of distal nephron cells.

466

467 A separate study performed RNA-sequencing analysis on dissected mandibular processes from double
468 conditional *Tfap2a/Tfap2b* mouse mutant embryos to find *Tfap2a/2b* target genes during branchial arch
469 patterning (Van Otterloo et al., 2018). Upon evaluating differentially expressed genes by Geneset

470 enrichment analyses (GSEAs) 'homeobox transcription factors' was identified as the number one over-
471 represented cluster. Further genetic workup of *Dlx*, *Msx*, and *Emx* homeobox gene families established
472 these factors as major network targets under the control of *Tfap2a/b* during branchial arch development.
473 Performing RNA-profiling and GSEAs of *tfap2a*-deficient developing nephrons would aid in pinpointing
474 molecular pathways for future workup. The majority of differentially expressed genes in *Tfap2a/2b* murine
475 branchial arches were associated with regions corresponding to poised histone marks, supporting a direct
476 mode of regulation (Van Otterloo et al., 2018). However, it remains a possibility that *tfap2a* regulates
477 transcription in nephron precursors via an intermediate factor or chromatin modifier as part of the GRN. In
478 further support of an indirect mode of regulation, *Tfap2a* acts as a tissue-specific pioneer factor in the
479 epididymis to modify chromatin structure activating androgen receptor signaling (Pihlajamaa et al., 2014).
480 We speculate that *Tfap2a/b* operates both directly and indirectly to regulate expression of GRN
481 components responsible for terminal differentiation of distal nephron tubules. To entertain this prospect,
482 ATAC-seq and ChIP-seq methods could be installed in the future to determine differential chromatin
483 accessibility in *tfap2a*-deficient renal progenitors.

484

485 *Tfap2a* has roles in induction, early specification, patterning, cell survival, and differentiation depending on
486 the tissue type (Eckert et al., 2005). In neural crest, *tfap2a* plays dual roles in early development and later
487 differentiation events. *tfap2a* and *foxd3* establish proper Bmp and Wnt signaling, which is required for early
488 neural crest induction (de Croze et al., 2011; Bhat et al., 2012). In *tfap2a*-deficiency, migratory neural crest
489 cells undergo increased apoptosis, indicating *tfap2a* is necessary for survival. It is speculated that because
490 these cells cannot differentiate properly, they undergo apoptosis (Knight et al., 2003). Further, *tfap2a* is
491 required for the specification and differentiation of inner ear neurons (Kantarci et al., 2015). *tfap2a* is also a
492 necessary element for establishing preplacodal ectoderm competence and specification of ectoderm
493 lineages (Bhat et al., 2012; Kwon et al., 2010). The concerted action of *tfap2a* and *phox2a* promote the
494 differentiation of noradrenergic neurons in the central nervous system (Holzschuh et al., 2003). *tfap2a* also
495 stimulates pathways that promote melanocyte terminal differentiation and survival (Van Otterloo et al.,
496 2010; Seberg et al., 2017). *tfap2a* and *tfap2b* are required for the survival of sympathetic progenitors and
497 differentiated sympathetic neurons (Schmidt et al., 2011). Differentiation of amacrine cells during
498 retinogenesis is also induced by *tfap2a* and *tfap2b* (Bassett et al., 2012; Jin et al., 2015), and they function
499 redundantly and non-autonomously to regulate cartilage patterning by modulating Fgf signals in the
500 pharyngeal ectoderm (Knight et al., 2005). Interestingly, in the developing mouse kidney, *Tfap2b* is
501 required for the maintenance and survival of renal epithelium (Moser et al., 1997). In *Tfap2b* null mice,
502 distal tubules and collecting duct cells undergo a massive wave of apoptosis. Additionally, histology
503 revealed mutant kidneys possess numerous cysts in the distal tubules and collecting ducts. Anti-apoptotic
504 genes *bcl-X_L*, *bcl-w*, and *bcl2* are strongly downregulated, supporting the idea that *Tfap2b* programs cell
505 survival during embryogenesis. However, in our *trm* mutant, we are confident cell death does not
506 contribute to the decreased solute transporter expression observed. Upon acridine orange analysis, we

507 saw no obvious increase in the number of dying cells. Further, we observe no gaps in the nephron tubule
508 as a result of dying cells, as indicated by continuous expression of *cdh17* expression at 24 hpf or at later
509 time points. Our data strongly suggests that the main function of *tfap2a* and *tfap2b* during nephron
510 development is not specification, patterning, or survival, but rather to promote terminal differentiation of
511 distal nephron epithelium.

512

513 Based on recent studies in the zebrafish model, upstream candidates for regulating *tfap2a* and *tfap2b* may
514 occupy the prostaglandin signaling pathway, which is essential to control the balance of DE and DL
515 territories during zebrafish nephrogenesis (Poureetezadi et al., 2016), or include transcription factors like
516 *mecom*, *tbx2a/2b*, or *emx1* that regulate DL development (Li et al., 2014; Drummond et al., 2016; Morales
517 et al., 2018). Additional network candidates which may crosstalk with *tfap2a/2b* include *sall1* and *sox11*. In
518 mammals *Sall1* has been found to be a critical factor in the development of the thick ascending limb (TAL),
519 which is the segment analogous to the zebrafish DE region (Basta et al., 2017). *Sall1* mutants exhibited
520 significantly decreased expression of *Kcnj1* (*Romk2*), *Slc12a1*, *Irx2*, and *Pou3f3*, among other major loop
521 of Henle and distal lineage genes. Immunohistochemistry analysis revealed a near total loss of *Slc12a1*⁺
522 loop of Henle structures in the inner medulla of mutant embryos. In the murine kidney, *Sox11* is also
523 necessary for proper loop of Henle ontogeny (Neirijnck et al., 2018). *Sox11*-deficient kidneys have
524 significantly reduced expression of *Slc12a1*, *Irx1*, and *Irx2*. *Sall1* and *Sox11* are excellent candidates to
525 situate in the *Tfap2a* GRN, due to their involvement in the DE/TAL segment development. Additionally,
526 *Sall1*, *Sox11*, and *tfap2a* mutations all reduce *Irx* gene expression, suggesting they may act in the same
527 pathway.

528

529 Knowledge about the terminal differentiation programs of each nephron segment has central importance
530 for understanding kidney disease and to advance regenerative medicine. Human BOFS is associated with
531 the occurrence of dysplastic kidneys, but the underlying mechanisms are not known. Our zebrafish *trm*
532 mutant provides an opportunity to model aspects of BOFS at the molecular level of the nephron. With
533 regard to kidney engineering, current groups face major challenges of generating mature, differentiated
534 nephron structures in kidney organoid cultures (Hariharan et al., 2015; Chambers et al., 2016; Oxburgh et
535 al., 2017; Takasato et al., 2017). However, growing mouse and particularly human kidney organoids is an
536 immensely promising technology to study kidney development, model renal disease, and perform
537 nephrotoxicity assays (Morizane and Bonventre, 2017). Reconstructing the mammalian nephron requires
538 understanding the correct signals to guide stem cells down the appropriate differentiation paths to
539 generate highly specialized compartments of cells. While terminally differentiated nephrons have yet to be
540 achieved in organoid cultures, the discovery of terminal differentiation factors, like *tfap2a* and *tfap2b*, can
541 herald progress in this crucial aspect of the kidney organoid field. In sum, our work indicates that further
542 elucidation of the *Tfap2a/TFAP2A* gene regulatory network in zebrafish, murine, and human nephron
543 progenitors can shed valuable insights into nephron differentiation and congenital renal disease.

544 **Materials and Methods**

545

546 **Ethics statement and zebrafish husbandry**

547 Adult zebrafish were maintained in the Center for Zebrafish Research at the University of Notre Dame
548 Freimann Life Science Center. All studies were performed and supervised with by the University of Notre
549 Dame Institutional Animal Care and Use Committee (IACUC), under protocol numbers 13-021 and 16-025.
550 Tübingen strain animals were used for WT experiments. Zebrafish embryos were raised in E3 embryo
551 media, staged, and fixed as described (Kimmel et al., 1995).

552

553 **Whole mount and fluorescent *in situ* hybridization (WISH, FISH)**

554 WISH and FISH were performed as described (Marra et al., 2017; Brend and Holley, 2009; Lengerke et al.,
555 2011; Cheng et al., 2014) with antisense RNA probes. Probes were synthesized using IMAGE clone
556 template plasmids for *in vitro* transcription (Wingert et al., 2007; Wingert and Davidson, 2011).
557 Digoxigenin-labeled probes consist of: *wt1b*, *slc20a1a*, *slc12a1*, *dlx2a*, *romk2*, *tfap2a*, *tfap2b*, *clcnk*,
558 *slc12a3*, *slc9a3*, *cdh17*, *hnf1ba*, *irx3b*, *irx1a*, *trpm7*, and *stc1*. Fluorescein-labeled probes consist of:
559 *tfap2b*, *slc12a1*, *slc12a3*, *tfap2a*. For all gene expression studies, each analysis was performed in triplicate
560 with sample size of at least n=20 for each replicate. Representative animals were imaged and absolute
561 length measurements were collected.

562

563 **Whole mount immunofluorescence (IF)**

564 Whole mount IF studies were completed as described (McC Campbell et al., 2015). To assess Tfap2a
565 protein expression, anti-tfap2a (1:50) (LifeSpan Biosciences) and anti-goat secondary antibody were used.
566 To analyze proliferation, anti-phospho-Histone H3 (1:200) (Millipore), and anti-rabbit secondary antibody
567 (Alexa Fluor, Invitrogen) were used (Kroeger et al., 2017). For cilia studies, anti-acetylated α -tubulin
568 (1:400) and anti-mouse secondary antibody (Alexa Fluor, Invitrogen) were used (Marra et al., 2017).
569 Monoclonal α 6F anti-NaKATPase (1:35) (Developmental Studies Hybridoma Bank) and anti-aPKC (1:250)
570 (Santa-Cruz) were applied to embryos incubated in 0.003% PTU to prevent pigmentation and fixed in
571 Dent's solution (80% methanol, 20% DMSO) overnight at 4°C (Gerlach and Wingert, 2014). Anti-mouse
572 and anti-rabbit secondary antibodies (Alexa Fluor, Invitrogen) were used respectively. All fluorescently-
573 conjugated secondary antibodies were applied at a 1:500 dilution, and 4,6-diamidino-2-phenylindole
574 dihydrochloride (DAPI) (Invitrogen) was used to stain nuclei.

575

576 **Image acquisition and statistical analysis**

577 A Nikon Eclipse Ni with DS-Fi2 camera was used to image WISH samples. A Nikon C2 confocal
578 microscope was used to image whole mount FISH and IF samples. The polyline tool in Nikon Elements
579 imaging software was used to measure gene expression domains. A minimum of 3 representative samples

580 for each control and experimental group were imaged and measured. Averages and standard errors were
581 calculated. Unpaired t-tests or one-way ANOVA tests were completed for statistical analyses.

582

583 **Mutagenesis, whole genome sequencing and genotyping**

584 WT zebrafish were exposed to ethylnitrosurea and haploids generated as described (Kroeger et al., 2014).
585 Whole genome sequencing was performed as described (Leshchiner et al., 2012). Pools of 20 *trm* mutants
586 and 20 WT siblings were identified by WISH analysis for *slc12a1* (DE) expression. DNA isolation was
587 conducted using the DNAeasy blood and tissue kit (Qiagen). WGS results were interpreted by SNPtrack
588 software (Leshchiner et al., 2012; Ryan et al., 2013). Isolation of genomic DNA from individual *trm* animals
589 was performed and PCR amplification of the *tfap2a* locus was completed using the following primers:

590 forward 5'-

591 TTTGAACGCTGGCCACCGCCACCTCGCCCTACAATTATTGTTGGCTTGATTTAATTTGCACGTTTCGTTT

592 TTGATTTGTCCTTCTGAATTTACGTCTTTT-3' reverse 5'

593 AAATGTTTGGTTTTTCGTTTACCAGTTAAAATCCTACCGAAAGGCAAAGGAAATTAACAATTAACCACAG

594 CTCACATGAAGAAAATCTTTGTAATAGCCTT-3'. For all studies, *trm* mutants were confirmed by

595 genotyping and/or abrogated *dlx2* expression. The QIAquick PCR Purification Kit was used to purify PCR

596 product and sequenced with the forward primer by the University of Notre Dame Genomics Core Facility.

597 Genotyping of *hs:tfap2a* transgenic (*Tg(hsp70:tfap2a)^{x24}*, which was a generous gift from Bruce Riley, was

598 conducted by performing PCR amplification (34 cycles, 60°C annealing) of the transgene and running

599 product on a 1% agarose gel. The following primers were used: forward 5'-CTCCTCTCAATGACAGCTG-3'

600 reverse 5'-ATGGCGGTTGGAAGTCTGAA-3'.

601

602 **Overexpression experiments**

603 To activate the heat-shock inducible *tfap2a* transgene, heterozygous transgenic embryos were incubated

604 at 38°C for 30 minutes as described (Bhat et al., 2012; Kantarci et al., 2015). For rescue and gain-of-

605 function studies, transgenic embryos were heat shocked at the 8ss. For FISH gain-of-function studies,

606 transgenic embryos were heat shocked at the 10ss. For cRNA synthesis, the open reading frame of *tfap2a*

607 was subcloned into the pCS2 vector. The primers used for subcloning were: forward 5'-

608 GATCATCGATGCCGCCACCATGTTAGTGACAGTTTTTCCGCGATGGATC-3' reverse 5'-

609 GATCTCTAGATCACTTTCTGTGCTTCTCATCTTTGTCACC-3'. For *in vitro* transcription, *tfap2a* template

610 was linearized using Not1 restriction enzyme. Runoff reactions were performed using the mMessage

611 mMachine Sp6 kit (Ambion). 50 picograms (pg) of *tfap2a* cRNA were injected into 1 cell stage embryos.

612

613 **Morpholino knockdown and RT-PCR**

614 All morpholino oligonucleotides were synthesized by Gene Tools, LLC. *tfap2a* MO-splice (*tfap2a* MO4)

615 targets the exon 2 – intron 2 splice site: 5'-AGCTTTTCTTCTTACCTGAACATCT-3' [36]. *tfap2b* MO-splice

616 (*tfap2b* MO1) targets the exon 4 – intron 4 splice site: 5'-GCCATTTTTCGACTTCGCTCTGATC-3' (Knight

617 et al., 2005). *irx3b* MO-ATG (*irx3b* MO2) targets the start site: 5'-ATAGCCTAGCTGCGGGAGAGACATG-
618 3'. Morpholinos were solubilized in DNase/RNase free water to create 4mM stock solutions and stored at -
619 20°C. The stocks were diluted as follows for microinjection: *tfap2a*-MO 1:12, *tfap2b*-MO 1:10, *irx3b* MO
620 1:10. 1-cell stage embryos were injected with approximately 3 nl of morpholino. All splice-blocking MOs
621 were verified by RT-PCR. Transcript analysis of *tfap2a* and *tfap2b* splicing in WT, WT sibs, *trm* mutants,
622 *tfap2a* morphants, and *tfap2b* morphants was performed using RT-PCR (Galloway et al., 2008). In brief,
623 RNA was isolated from pools of about 20 embryos, cDNA was synthesized using random hexamers
624 (Superscript IV, Invitrogen), and PCR was performed with the following primers. *trm* mutant transcript
625 analysis: forward 5'-GCATTGCATCTAA-AGGGCAGACGAA-3' reverse 5'-
626 TAAGGGTCCTGAGACTGCGGATAGA- 3'. *tfap2a* MO-splice transcript analysis: forward 5'-
627 CCCTATCCATGGAATACCTCACTC-3' reverse 5'-GATTACA-GTTTGGTCTGGGATGTGA-3'. *tfap2b* MO-
628 splice transcript analysis: forward 5'-AGTGC-CTGAACGCGTCTCTGCTTGGT-3' reverse 5'-
629 TGACATTGCTGCCTTGCGTCTCC-3'. For *tfap2a* MO and *tfap2b* MO transcript analysis, bands were
630 gel-extracted, purified, and sequenced. For *trm* mutant transcript analysis, bands were gel-extracted,
631 purified, and cloned into the pGemTEasy vector (Promega), and minipreps were sequenced.
632

633 **Alcian blue staining and o-dianisidine staining**

634 Alcian blue cartilage staining was performed as previously described (Neuhauss et al., 1996). In brief,
635 larvae were fixed at 4 dpf for 2 hours at RT in 4% PFA. Larvae were bleached for 1 hour in 10% KOH, 30%
636 H₂O₂, 20% Tween diluted in distilled water. Samples were digested with proteinase K (10 mg/mL) diluted to
637 1X for 20 minutes. Samples were stained in 0.1% Alcian Blue (Sigma) dissolved in 70% ethanol / 5%
638 concentrated HCl overnight, shaking at RT in glass vials. Larvae were destained using acidic ethanol for 4
639 hours, dehydrated by an ethanol series, and stored in glycerol. O-Dianisidine staining was performed as
640 described on 4 dpf larvae to visualize blood and vasculature (Wingert et al., 2004).
641

642 **Acridine orange assay**

643 Acridine orange (AO; Sigma A6014; 100 X) staining was performed on WT and *trm* mutants to analyze cell
644 death (Kroeger et al., 2017; Westerfield, 193). In brief, a 50 X AO stock solution (250 µg/ml) was made. At
645 24 hpf, embryos were incubated in 1:50 AO solution (made from 50 X stock) diluted in 0.003% PTU/E3
646 media protected from light for 1 hour. Embryos were then washed three times with 0.003% PTU/E3, and
647 then imaged with a dissecting microscope under the GFP filter in 2% methylcellulose/0.02% tricaine.
648

649 **Dextran clearance assay**

650 To assess kidney function in WT and *trm* mutants, clearance assays using fluorescent 40 kDa dextran-
651 fluorescein (FITC) (Invitrogen) were completed. Embryos were treated with 0.003% PTU at 24 hpf. At 2
652 dpf, embryos were anesthetized with 0.02% tricaine and dextran-FITC was injected into circulation. Live

653 fluorescent imaging was performed 1 hour after injection and 24 hours after injection. Embryos were live-
654 imaged with a dissecting microscope under the GFP filter in methylcellulose/0.02% tricaine.
655

656 **Acknowledgements**

657 We thank Bruce Riley for sharing the inducible *hs:tfap2a* zebrafish transgenic with us. We thank the staffs
658 of the Department of Biological Sciences and the Center for Zebrafish Research at the University of Notre
659 Dame for their dedication and care of our zebrafish aquarium. Finally, we thank the members of our lab for
660 their support, discussions, and insights about this work.

661

662

663

664 **Competing Interests**

665 The authors declare no competing interests.

666

667

668

669 **Funding**

670 This work was supported by the National Institutes of Health [R01DK100237 to R.A.W.]. We are grateful to
671 Elizabeth and Michael Gallagher for a generous gift to the University of Notre Dame on behalf of their
672 family for the support of stem cell research. The funders had no role in the study design, data collection
673 and analysis, decision to publish, or manuscript preparation.

674

675

676

677 **Author Contributions**

678 BEC, GFG, KHC, EGC, IL and RAW performed experiments. BEC, GFG, KHC, EGC, WG and RAW
679 analyzed the results. BEC and RAW wrote and revised the paper.

680

681

682

683 **Data Availability**

684 All data related to the present study is provided within the figures and supplementary information.

685 **References**

686

687 **Alarcón, P., Rodríguez-Seguel, E., Fernández-González, A., Rubio, R., and Gómez-Skarmeta, J.L.**
688 (2008). A dual requirement for Iroquois genes during *Xenopus* kidney development. *Development* **135**,
689 3197-3207.

690

691 **Airik, R., and Kispert, A.** (2007). Down the tube of obstructive nephropathies: the importance of tissue
692 interactions during ureter development. *Kidney Int.* **72**, 1459-1467.

693

694 **Barrallo-Gimeno, A., Holzschuh, J., Driever, W., and Knapik, E.W.** (2003). Neural crest survival and
695 differentiation in zebrafish depends on mont blanc/tfap2a gene function. *Development* **131**, 1463-1477.

696

697 **Bassett, E.A., Korol, A., Deschamps, P.A., Buettner, R., Wallace, V.A., Williams, T., and West-Mays**
698 **J.A.** (2012). Overlapping expression patterns and redundant roles for AP-2 transcription factors in the
699 developing mammalian retina. *Dev. Dyn.* **241**, 814-829.

700

701 **Basta, J. M., Robbins L., Denner D. R., Kolar, G. R., and Rauchman, M. A.** (2017). Sall1-NuRD
702 interaction regulates multipotent nephron progenitors and is required for loop of Henle formation.
703 *Development* **144**, 3080-3094.

704

705 **Bhat, N., Kwon, H.J., and Riley, B.B.** (2012). A gene network that coordinates preplacodal competence
706 and neural crest specification in zebrafish. *Dev. Biol.* **373**, 107-117.

707

708 **Brend, T., and Holley, S.A.** (2009). Zebrafish whole mount high-resolution double fluorescent in situ
709 hybridization. *J. Vis. Exp.* **25**, pii1229.

710

711 **Brewer, S., and Williams, T.** (2004a) Loss of AP-2alpha impacts multiple aspects of ventral body wall
712 development and closure. *Dev. Biol.* **267**, 399-417.

713

714 **Brewer, S., Feng, W., Huang, J., Sullivan, S., and Williams, T.** (2004b) Wnt1-Cre-mediated deletion of
715 AP-2alpha causes multiple neural crest-related defects. *Dev. Biol.* **267**, 135-52.

716

717 **Chambers, J., McKee, R.A., Drummond, B.E., and Wingert R.A.** (2016). Evolving technology: creating
718 kidney organoids from stem cells. *AIMS Bioeng.* **3**, 305-318.

719

720 **Cheng, C.W., Hui, C., Strähle, U., and Cheng, S.H.** (2001). Identification and expression of zebrafish
721 Iroquois homeobox gene *irx1*. *Dev. Genes. Evol.* **211**, 442-444.

722

723 **Cheng, C.N., Li, Y., Marra, A.N., Verdun, V., and Wingert, R.A.** (2014). Flat mount preparation for
724 observation and analysis of zebrafish embryo specimens stained by whole mount in situ hybridization. *J.*
725 *Vis. Exp.* **89**, e51604.

726

727 **Cheng, C.N., and Wingert, R.A.** (2015). Nephron proximal tubule patterning and corpuscles of Stannius
728 formation are regulated by the *sim1a* transcription factor and retinoic acid in zebrafish. *Dev. Biol.* **399**, 100-
729 116.

730

731 **Chung, E., Deacon, P., and Park, J.S.** (2017). Notch is required for the formation of all nephron segments
732 and primes nephron progenitors for differentiation. *Development* **144**, 4530-4539.

733

734 **de Crozé, N., Maczkowiak, F., and Monsoro-Burq, A.H.** (2011). Reiterative AP2a activity controls
735 sequential steps in the neural crest gene regulatory network. *Proc. Natl. Acad. Sci. USA* **108**, 155-60.

736

737 **Desgrange, A., and Cereghini, S.** (2015). Nephron patterning: lessons from *Xenopus*, zebrafish and
738 mouse studies. *Cells* **4**, 483-499.

- 739
740 **Drake, K.A., Adam, M., Mahoney, R., and Potter, S.S.** (2018). Disruption of Hox9,10,11 function results
741 in cellular level lineage infidelity in the kidney. *Sci. Rep.* **8**, 6306.
742
743 **Dressler, G. R.** (2006). The cellular basis of kidney development. *Annu. Rev. Cell Dev. Biol.* **22**, 509-529.
744
745 **Drummond, B.E., Li, Y., Marra, A.N., Cheng, C.N., and Wingert, R.A.** (2016). The tbx2a/b transcription
746 factors direct pronephros segmentation and corpuscle of Stannius formation in zebrafish. *Dev. Biol.* **421**,
747 52-66.
748
749 **Eckert, D., Buhl, S., Weber, S., Jäger, R., and Schorle, H.** (2005). The AP-2 family of transcription
750 factors. *Genome Biol.* **6**, 246.
751
752 **Galloway, J.L., Wingert, R.A., Thisse, C., Thisse, B., and Zon, L.I.** (2008). Combinatorial regulation of
753 novel erythroid gene expression in zebrafish. *Exp. Hematol.* **36**, 424–432.
754
755 **Gerlach, G.F., and Wingert, R.A.** (2013) Kidney organogenesis in the zebrafish: insights into vertebrate
756 nephrogenesis and regeneration. *Wiley Interdiscip. Rev. Dev. Biol.* **2**, 559-585.
757
758 **Gerlach, G.F., and Wingert, R.A.** (2014). Zebrafish pronephros tubulogenesis and epithelial identity
759 maintenance are reliant on the polarity proteins Prkc iota and zeta. *Dev. Biol.* **396**,183-200.
760
761 **Greco, D., Zellmer, E., Zhang, Z., and Lewis, E.** (1995). Transcription factor AP-2 regulates expression
762 of the dopamine beta-hydroxylase gene. *J. Neurochem.* **65**, 510-516.
763
764 **Green, R.M., Feng, W., Phang, T., Fish, J.L., Li, H., Spritz, R.A., Marcucio, R.S., Hooper, J.,**
765 **Jamniczky, H., Hallgrímsson, B., et al.** (2014). Tfp2a-dependent changes in mouse facial morphology
766 result in clefting that can be ameliorated by a reduction in Fgf8 gene dosage. *Dis. Model Mech.* **8**, 31-43.
767
768 **Handwerger, S.** (2009). New insights into the regulation of human cytotrophoblast cell differentiation. *Mol.*
769 *Cell Endocrinol.* **323**, 94-104.
770
771 **Hariharan, K., Kurtz, A., and Schmidt-Ott, K.M.** (2015). Assembling kidney tissues from cells: the long
772 road from organoids to organs. *Front. Cell Dev. Biol.* **3**, 70.
773
774 **Hilger-Eversheim, K., Moser, M., Schorle, H., and Buettner, R.** (2000). Regulatory roles of AP-2
775 transcription factors in vertebrate development, apoptosis and cell-cycle control. *Gene* **260**,1-12.
776
777 **Hoffman, T.L., Javier, A.L., Campeau, S.A., Knight, R.D., and Schilling, T.F.** (2007). Tfp2 transcription
778 factors in zebrafish neural crest development and ectodermal evolution. *J. Exp. Zool. B Mol. Dev. Evol.*
779 **308**, 679-691.
780
781 **Holzschuh, J., Barrallo-Gimeno, A., Ettl, A.K., Durr, K., Knapik, E.W., Driever, W.** (2003).
782 Noradrenergic neurons in the zebrafish hindbrain are induced by retinoic acid and require tfap2a for
783 expression of the neurotransmitter phenotype. *Development* **130**, 5741-5754.
784
785 **Jin, K., Jiang, H., Xiao, D., Zou, M., Zhu, J., and Xiang, M.** (2015). Tfp2a and 2b act downstream of
786 Ptf1a to promote amacrine cell differentiation during retinogenesis. *Mol. Brain.* **8**, 28.
787
788 **Kantarci, H., Edlund, R.K., Groves, A.K., and Riley B.B.** (2015). Tfp2a promotes specification and
789 maturation of neurons in the inner ear through modulation of Bmp, Fgf and notch signaling. *PLoS Genet.*
790 **11**, e1005037.
791
792 **Kerber, B., Monge, I., Mueller, M., Mitchell, P.J., and Cohen, S.M.** (2001). The AP-2 transcription factor
793 is required for joint formation and cell survival in Drosophila leg development. *Development* **128**, 1231-8.

794

795 **Kim, H.S., Hong, S.J., LeDoux, M.S., and Kim, K.S.** (2001). Regulation of the tyrosine hydroxylase and
796 dopamine beta-hydroxylase genes by the transcription factor AP-2. *J. Neurochem.* **76**, 280-294.

797

798 **Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., and Schilling, T.F.** (1995). Stages of
799 embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.

800

801 **Knight, R.D., Nair, S., Nelson, S.S., Afshar, A., Javidan, Y., Geisler, R., Rauch, G.J., and Schilling,**
802 **T.F.** (2003). lockjaw encodes a zebrafish tfap2a required for early neural crest development. *Development*
803 **130**, 5755-68.

804

805 **Knight, R.D., Javidan, Y., Zhang, T., Nelson, S., and Schilling, T.F.** (2005). AP2-dependent signals
806 from the ectoderm regulate craniofacial development in the zebrafish embryo. *Development* **132**, 3127-
807 3138.

808

809 **Kroeger, P.T. Jr., Poureetezadi, S.J., McKee, R., Jou, J., Miceli, R., and Wingert, R.A.** (2014).
810 Production of haploid zebrafish embryos by in vitro fertilization. *J. Vis. Exp.* **89**, e51708.

811

812 **Kroeger, P.T. Jr., Drummond, B.E., Miceli, R., McKernan, M., Gerlach, G.F., Marra, A.N., Fox, A.,**
813 **McCampbell, K.K., Leshchiner, I., Rodriguez-Mari, A., BreMiller, R., et al.** (2017). The zebrafish kidney
814 mutant zeppelin reveals that brca2/fancd1 is essential for pronephros development. *Dev. Biol.* **428**,148-
815 163.

816

817 **Kwon, H.J., Bhat, N., Sweet, E.M., Cornell, R.A., and Riley, B.B.** (2010). Identification of early
818 requirements for preplacodal ectoderm and sensory organ development. *PLoS Genet.* **6**, e1001133.

819

820 **Lengerke, C., Wingert, R., Beeretz, M., Grauer, M., Schmidt, A.G., Konantz, M., Daley, G.Q., and**
821 **Davidson, A.J.** (2011). Interactions between Cdx genes and retinoic acid modulate early cardiogenesis.
822 *Dev. Biol.* **163**,134–142.

823

824 **Leshchiner, I., Alexa, K., Kelsey, P., Adzhubei, I., Austin-Tse, C.A., Cooney, J.D., Anderson, H.,**
825 **King, M.J., Stottmann, R.W., Garnaas, M.K., et al.** (2012). Mutation mapping and identification by whole-
826 genome sequencing. *Genome Res.* **22**, 1541-1548.

827

828 **Li, Y., Cheng, C.N., Verdun, V.A., and Wingert, R.A.** (2014). Zebrafish nephrogenesis is regulated by
829 interactions between retinoic acid, mecom, and Notch signaling. *Dev. Biol.* **386**, 111-122.

830

831 **Li, W., and Cornell, R.A.** (2007). Redundant activities of Tfap2a and Tfap2c are required for neural crest
832 induction and development of other non-neural ectoderm derivatives in zebrafish embryos. *Dev. Biol.* **304**,
833 338-54.

834

835 **Lindström, N.O., Lawrence, M.L., Burn, S.F., Johansson, J.A., Bakker, E.R.M., Ridgway, R.A.,**
836 **Chang, C.H., Karolak, M.J., Oxburgh, L., Headon, D.J., et al.** (2015). Integrated B-Catenin, BMP, PTEN,
837 and Notch signalling patterns the nephron. *Elife* **3**, e04000.

838

839 **Lindström, N.O., McMahon, J.A., Guo, J., Tran, T., Guo, Q., Rutledge, E., Parvez, R.K., Saribekyan,**
840 **G., Schuler, R.E., Liao, C., et al.** (2018a). Conserved and divergent features of human and mouse kidney
841 organogenesis. *J. Am. Soc. Nephrol.* **29**, 785-805.

842

843 **Lindström, N.O., De Sena Brandine, G., Tran, T., Ransick, A., Suh, G., Guo, J., Kim, A.D., Parvez,**
844 **R.K., Ruffins, S.W., Rutledge, E.A., et al.** (2018b). Progressive recruitment of mesenchymal progenitors
845 reveals a time-dependent process of cell fate acquisition in mouse and human nephrogenesis. *Dev. Cell.*
846 **45**, 651-660.

- 847 **Magella, B., Mahoney, R., Adam, M., and Potter, S.S.** (2018). Reduced Abd-B Hox function during
848 kidney development results in lineage infidelity. *Dev. Biol.* **438**, 84-93.
- 849
- 850 **Marra, A.N., and Wingert, R.A.** (2014). Roles of Iroquois transcription factors in kidney development. *Cell*
851 *Dev. Biol.* **3**, 1000131.
- 852
- 853 **Marra, A.N., and Wingert, R.A.** (2016). Epithelial cell fate in the nephron tubule is mediated by the ETS
854 transcription factors *etv5a* and *etv4* during zebrafish kidney development. *Dev. Biol.* **411**, 231-245.
- 855
- 856 **Marra, A.N., Li, Y., and Wingert, R.A.** (2016). Antennas of organ morphogenesis: the roles of cilia in
857 vertebrate kidney development. *Genesis* **54**, 457-469.
- 858
- 859 **Marra, A.N., Ulrich, M., White, A., Springer, M., and Wingert, R.A.** (2017). Visualizing multiciliated cells
860 in the zebrafish through a combined protocol of whole mount fluorescent in situ hybridization and
861 immunofluorescence. *J. Vis. Exp.* **129**, e56261.
- 862
- 863 **McCampbell, K.K., Springer, K.N., and Wingert, R.A.** (2015). Atlas of cellular dynamics during zebrafish
864 adult kidney regeneration. *Stem Cells Int.* **2015**, 547636.
- 865
- 866 **McKee, R., Gerlach, G.F., Jou, J., Cheng, C.N., and Wingert, R.A.** (2014). Temporal and spatial
867 expression of tight junction genes during zebrafish pronephros development. *Gene Expr. Patterns* **16**, 104-
868 113.
- 869
- 870 **Milunsky, J.M., Maher, T.A., Zhao, G., Roberts, A.E., Stalker, H.J., Zori, R.T., Burch, M.N., Clemens,
871 M., Mulliken, J.B., Smith, R., et al.** (2008). TFAP2A mutations result in branchio-oculo-facial syndrome.
872 *Am. J. Hum. Genet.* **82**,1171-1177.
- 873
- 874 **Morales, E.M., Handa, N., Drummond, B.E., Chambers, J.M., Marra, A.N., Addiego, A., and Wingert,
875 R.A.** (2018). Homeogene *emx1* is required for nephron distal segment development in zebrafish. *Sci.*
876 *Reports* in press.
- 877
- 878 **Moser, M., Rüschoff, J., and Buettner, R.** (1997a). Comparative analysis of AP-2 alpha and AP-2 beta
879 gene expression during murine embryogenesis. *Dev. Dyn.* **208**, 115-24.
- 880
- 881 **Moser, M., Pscherer, A., Roth, C., Becker, J., Mücher, G., Zerres, K., Dixkens, C., Weis, J., Guay-
882 Woodford, L., Buettner R., et al.** (1997b). Enhanced apoptotic cell death of renal epithelial cells in mice
883 lacking transcription factor AP-2beta. *Genes Dev.***11**, 1938-48.
- 884
- 885 **Naylor, R.W., Przepiorski, A., Ren, Q., Yu, J., and Davidson, A.J.** (2013). HNF1β is essential for
886 nephron segmentation during nephrogenesis. *J. Am. Soc. Nephrol.* **24**, 77-87.
- 887
- 888 **Naylor, R.W., Qubisi, S.S., and Davidson, A.J.** (2017). Zebrafish pronephros development. *Results*
889 *Probl. Cell Differ.* **60**, 27-53.
- 890
- 891 **Neirijnck, Y., Reginensi, A., Renkema, K.Y., Massa, F., Kozlov, V.M., Dhib, H., Bongers, E.M.H.F.,
892 Feitz, W.F., van Eerde, A.M., Lefebvre, V., et al.** (2018). Sox11 gene disruption causes congenital
893 anomalies of the kidney and urinary tract (CAKUT). *Kidney Int.* **93**, 1142-1153.
- 894
- 895 **Neuhauss, S.C., Solnica-Krezel, L., Schier, A.F., Zwartkruis, F., Stemple, D.L., Malicki, J., Abdelilah,
896 S., Stainier, D.Y., and Driever W.** (1996). Mutations affecting craniofacial development in zebrafish.
897 *Development* **123**, 357-67.
- 898
- 899 **O'Brien, E.K., d'Alençon, C., Bonde, G., Li, W., Schoenebeck, J., Allende, M.L., Gelb, B.D., Yelon, D.,
900 Eisen, J.S., and Cornell, R.A.** (2004). Transcription factor Ap-2alpha is necessary for development of

- 901 embryonic melanophores, autonomic neurons and pharyngeal skeleton in zebrafish. *Dev. Biol.* **265**, 246-
902 261.
- 903
- 904 **Oxburgh, L., Carroll, T.J., Cleaver, O., Gossett, D.R., Hoshizaki, D.K., Hubbell, J.A., Humphreys,**
905 **B.D., Jain, S., Jensen, J., Kaplan, D.L., et al.** (2017). (Re)Building a Kidney. *J. Am. Soc. Nephrol.* **28**,
906 1370-1378.
- 907
- 908 **Pfisterer, P., Ehlermann, J., Hegen, M., and Schorle, H.** (2001). A subtractive gene expression screen
909 suggests a role of transcription factor AP-2 alpha in control of proliferation and differentiation. *J. Biol.*
910 *Chem.* **277**, 6637-44.
- 911
- 912 **Pihlajamaa, P., Sahu, B., Lyly, L., Aittomäki, V., Hautaniemi, S., and Jänne, O.A.** (2014). Tissue-
913 specific pioneer factors associate with androgen receptor cistromes and transcription programs. *EMBO J.*
914 **33**, 312-26.
- 915
- 916 **Poureetezadi, S.J., Cheng, C.N., Chambers, J.M., Drummond, B.E., and Wingert, R.A.** (2016).
917 Prostaglandin signaling regulates nephron segment patterning of renal progenitors during zebrafish kidney
918 development. *Elife* **5**, pii:e17551.
- 919
- 920 **Poureetezadi, S.J., and Wingert, R.A.** (2016) Little fish, big catch: zebrafish as a model for kidney
921 disease. *Kidney Int.* **89**, 1204-1210.
- 922
- 923 **Reggiani, L., Raciti, D., Airik, R., Kispert, A., and Brändli, A.W.** (2007). The prepattern transcription
924 factor *lrx3* directs nephron segment identity. *Genes Dev.* **21**, 2358-2370.
- 925
- 926 **Ryan, S., Willer, J., Marjoram, L., Bagwell, J., Mankiewicz, J., Leshchiner, I., Goessling, W., Bagnat,**
927 **M., and Katsanis, N.** (2013). Rapid identification of kidney cyst mutations by whole exome sequencing in
928 zebrafish. *Development* **140**, 4445-4451.
- 929
- 930 **Satoda, M., Zhao, F., Diaz, G.A., Burn, J., Goodship, J., Davidson, H.R., Pierpont, M.E., and Gelb**
931 **B.D.** (2000). Mutations in *TFAP2B* cause Char syndrome, a familial form of patent ductus arteriosus. *Nat.*
932 *Genet.* **25**, 42-6.
- 933
- 934 **Saxen, L.** (1987). *Organogenesis of the kidney*. Cambridge, UK: Cambridge University Press.
- 935
- 936 **Schedl, A.** (2007). Renal abnormalities and their developmental origin. *Nat. Rev. Genet.* **8**, 791-802.
- 937
- 938 **Schmidt, M., Huber, L., Majdazari, A., Schütz, G., Williams, T., and Rohrer H.** (2011). The transcription
939 factors AP-2 β and AP-2 α are required for survival of sympathetic progenitors and differentiated
940 sympathetic neurons. *Dev. Biol.* **355**, 89-100.
- 941
- 942 **Schorle, H., Meier, P., Buchert, M., Jaenisch, R., and Mitchell, P.J.** (1996). Transcription factor AP-2
943 essential for cranial closure and craniofacial development. *Nature* **381**, 235-8.
- 944
- 945 **Seberg, H.E., Van Otterloo, E., Loftus, S.K., Liu, H., Bonde, G., Sompallae, R., Gildea, D.E., Santana,**
946 **J.F., Manak, J.R., Pavan, W.J., et al.** (2017). *TFAP2* paralogs regulate melanocyte differentiation in
947 parallel with *MITF*. *PLoS Genet.* **13**, e1006636.
- 948
- 949 **Song, R., and Yosypiv, I.V.** (2011). Genetics of congenital anomalies of the kidney and urinary tract.
950 *Pediatr. Nephrol.* **26**, 353-364.
- 951
- 952 **Sugano, Y., Cianciolo Cosentino, C., Loffing-Cueni, D., Neuhauss, S.C.F., and Loffing, J.** (2017).
953 Comparative transcriptomic analysis identifies evolutionarily conserved gene products in the vertebrate
954 renal distal convoluted tubule. *Pflugers Arch.* **469**, 859-867.
- 955

- 956 **Takasato, M., and Little, M.H.** (2017). Making a kidney organoid using the directed differentiation of
957 human pluripotent stem cells. *Methods Mol. Biol.* **1597**, 195-206.
958
- 959 **Van Otterloo, E., Li, W., Bonde, G., Day, K.M., Hsu, M.Y., and Cornell, R.A.** (2010). Differentiation of
960 zebrafish melanophores depends on transcription factors AP2 alpha and AP2 epsilon. *PLoS Genet.* **6**,
961 e1001122.
962
- 963 **Van Otterloo, E., Li, H., Jones, K.L., and Williams, T.** (2018). AP-2 α and AP-2 β cooperatively
964 orchestrate homeobox gene expression during branchial arch patterning. *Development* **145**, pii:
965 dev157438.
966
- 967 **Wang, I.J., Tsai, R.J., Yeh, L.K., Tsai, R.Y., Hu, F.R., and Kao, W.W.** (2011). Changes in corneal basal
968 epithelial phenotypes in an altered basement membrane. *PLoS One* **6**, e14537.
969
- 970 **Wang J, Ji W, Zhu D, Wang W, Chen Y, Zhang Z, and Li F.** (2018). Tfp2b mutation in mice results in
971 patent ductus arteriosus and renal malformation. *J. Surg. Res.* **227**, 178-185.
972
- 973 **Westerfield, M.** (1993). *The Zebrafish Book*. Eugene, USA: University of Oregon Press.
974
- 975 **Wingert, R.A., Brownlie, A., Galloway, J.L., Dooley, K., Fraenkel, P., Axe, J.L., Davidson, A.J., Barut,**
976 **B., Noriega, L., Sheng, X., et al.** (2004). The chianti zebrafish mutant provides a model for erythroid-
977 specific disruption of transferrin receptor 1. *Development* **131**, 6225-6235.
978
- 979 **Wingert, R.A., Selleck, R., Yu, J., Song, H.D., Chen, Z., Song, A., Zhou, Y., Thisse, B., Thisse, C.,**
980 **McMahon, A.P., et al.** (2007). The cdx genes and retinoic acid control the positioning and segmentation of
981 the zebrafish pronephros. *PLoS Genet.* **3**, 1922-1938.
982
- 983 **Wingert, R.A., and Davidson, A.J.** (2008). The zebrafish pronephros: a model to study nephron
984 segmentation. *Kidney Int.* **73**, 1120-1127.
985
- 986 **Wingert, R.A., and Davidson A.J.** (2011). Zebrafish nephrogenesis involves dynamic spatiotemporal
987 expression changes in renal progenitors and essential signals from retinoic acid and irx3b. *Dev. Dyn.* **240**,
988 2011-2027.
989
- 990 **Zhang, J., Hagopian-Donaldson, S., Serbedzija, G., Elsemore, J., Plehn-Dujowich, D., McMahon,**
991 **A.P., Flavell, R.A., and Williams, T.** (1996). Neural tube, skeletal and body wall defects in mice lacking
992 transcription factor AP-2. *Nature* **381**, 238-241.
993
- 994 **Zhang, J., Yuan, S., Vasilyev, A., and Amin Arnaut, M.** (2015). The transcriptional coactivator Taz
995 regulates proximodistal patterning of the pronephric tubule in zebrafish. *Mech. Dev.* **3**, 328-335.

996 **Figure Legends**

997

998 **Figure 1: Forward genetic screen reveals *tfap2a* is necessary for nephrogenesis in the developing**

999 **zebrafish pronephros. A.** Schematic depicts lateral and dorsal views of fully segmented pronephros in 24
1000 hpf zebrafish embryo (P: podocytes, N: neck, PCT: proximal convoluted tubule, DE: distal early segment,
1001 CS: corpuscle of Stannius, DL: distal late segment, CD: collecting duct). **B.** Screening approach by WISH
1002 of alternating nephron compartment markers *wt1b/slc20a1a/slc12a1* (P/PCT/DE) in WT and *trm* at 24 hpf.
1003 Scale bar = 70µm. **C.** SNPtrack results from whole genome sequencing concentration of SNPs at
1004 chromosome 24. Confirmation of G → A *tfap2a* mutation by direct PCR sequencing of *trm*^{-/-} embryos. Exon
1005 diagram of *tfap2a* depicts 3 alternative spliceoforms (pink, cyan, orange lines). Black *'s indicate
1006 alternative start sites. *tfap2a* MO-splice (blue) targets 3' end of exon 2. *tfap2a*^{m819} lesion (red x) generates
1007 stop codon in exon 5. *trm* G > A mutation (red) maps to 3' end of exon 1c. Green letters indicate conserved
1008 splice residues. RT-PCR primers used for RT-PCR analysis flank intron 1-2 (purple arrows). **D.** RT-PCR
1009 analysis of control (WT) embryos, WT *trm* siblings, and *trm*^{-/-}. Mutant bands are labeled 1-4 in green. Table
1010 indicates predicted genetic consequence from sequencing the mutant bands. (TAD:transcriptional
1011 activation domain, DBD:DNA binding domain). **E.** Failure to complement revealed by WISH analysis of
1012 *dlx2a* (pharyngeal arches outlined in white) and *romk2* (DE) in WT and *trm*^{+/-} x *tfap2a*^{m819+/-} compound
1013 mutants. Scale bars = 70 µm, 35 µm. **F.** Live imaging at 4 dpf reveals abnormal craniofacial cartilage
1014 (black arrowhead) and pericardial edema (blue arrowhead) in *trm*^{-/-} and *tfap2a* morphants. Scale bar = 200
1015 µm. **G.** Alcian Blue cartilage staining in WT and *trm*^{-/-} at 4 dpf. Gaping jaw phenotype indicated by black
1016 arrowhead. Black dotted lines are utilized to trace Meckel's cartilage. Scale bar = 100 µm. **H.** Whole mount
1017 IF of Tfap2a protein in WT and *trm*^{-/-} at 24 hpf. White dotted lines delineate pronephric tubule. Cyan box
1018 denotes 40x optical zoom. Scale bar = 30 µm.

1019

1020

1021 **Figure 2: *tfap2a* and *tfap2b* transcripts are expressed in dynamic overlapping domains in**

1022 **developing nephrons, where *tfap2a* acts upstream of *tfap2b*.** **A.** WISH of WT *tfap2a* and *tfap2b*
1023 expression (purple) at the 10, 15, 20, 24, and 28 ss. *smyh1* (red) was used to mark somites. Black boxes
1024 indicate *tfap2* expression domains within developing renal progenitors. Scale bar = 200 µm. **B.** Double
1025 FISH of WT *tfap2a* (green) and *tfap2b* (red) transcript expression at 10 ss (flat mount), 20 ss, and 28 ss
1026 (lateral views). DAPI (blue) labels nuclei. White arrowheads demarcate cellular regions of overlapping
1027 transcripts within the pronephros. Scale bar = 70 µm. **C.** Double FISH of *slc12a1* (pink) and *tfap2a* (green)
1028 in WT embryo at 24 hpf. White box indicates area featured in bottom panel (60x z-stack). White dotted line
1029 outlines nephron tubule. DAPI (blue) labels nuclei. Scale bars = 70 µm, 10 µm. **D.** WISH analysis of *tfap2b*
1030 expression in WT and *trm*^{-/-}. Arrowhead indicates differential hindbrain expression of *tfap2b*. Black box
1031 designates *tfap2b* expression within pronephros. Scale bars = 100 µm, 50 µm. **E.** WISH analysis of *tfap2a*
1032 expression in WT and *tfap2b* MO. Black box designates *tfap2a* expression within pronephros. Scale bars =

1033 100 μm , 50 μm . **F.** Quantification of absolute length measurements of *tfap2b* expression domain within
1034 pronephros. **G.** Quantification of absolute length measurements of *tfap2a* expression domain within
1035 pronephros. $n = 3$ for each control and test group. Absolute measurements (in microns) were analyzed by
1036 unpaired t-tests. Data are represented as \pm SD. *** $p < 0.001$, N.S. = not significant.

1037

1038

1039 **Figure 3: *tfap2a* and *tfap2b* function redundantly to activate distal nephron solute transporter**
1040 **signature. A.** WT and *trm*^{-/-} embryos were microinjected with combinations of *tfap2a* and *tfap2b* splice-
1041 MOs. WISH was used to stain embryos for *slc12a1* (DE, purple), *slc12a3* (DL, red), and *clcnk* (Pandistal,
1042 purple) at 24 hpf. Black bars indicate WT marker domains. Scale bar = 35 μm . **B.** Quantification of
1043 absolute length of *slc12a1* expression domain. **C.** Quantification of absolute length measurements of
1044 *slc12a3* expression domain. **D.** Quantification of absolute length measurements of *clcnk* expression
1045 domain. $n \geq 4$ for each control and test group. Measurements were compared by ANOVA. Data are
1046 represented as \pm SD. * $p < 0.05$, ** $p < 0.01$, Green brackets indicate not statistically significant.

1047

1048

1049 **Figure 4: *tfap2a* is necessary and sufficient to drive the DE gene expression program. A.** *trm*^{-/-}
1050 */hs:tfap2a* and *hs:tfap2a* were heat-shocked at the 8 ss to overexpress WT Tfp2a protein for rescue and
1051 gain of function studies. WT embryos were microinjected with *tfap2a* cRNA for an independent gain of
1052 function studies. Control and experimental samples subjected to WISH analysis for *romk2* (DE) expression
1053 at 24 hpf. Black bar indicates WT *romk2* domain. Scale bars = 70 μm . **B.** Quantification of absolute length
1054 measurements of *romk2* expression domain. $n \geq 4$ for each control and test group. Measurements were
1055 compared by ANOVA. Data are represented as \pm SD. ** $p < 0.01$, Green brackets indicate not statistically
1056 significant. HS+ (red) signifies application of heat-shock. HS- (black) indicates no heat-shock. +*tfap2a*
1057 cRNA (blue) represents microinjection of *tfap2a* capped RNA at the 1-cell stage. **C.** Double FISH analysis
1058 of *slc12a1* (DE, green) and *slc12a3* (DL, red) in WT and heat shock-treated *hs:tfap2a* embryos at 24 hpf.
1059 Scale bar = 20 μm . White box indicates area imaged at higher (60X) objective in C'. **C'.** DAPI only (above)
1060 and merged channels (below). Dotted white line outlines a single cell coexpressing *slc12a1* and *slc12a3*
1061 transcripts. **D.** Double FISH analysis of *slc9a3* (panproximal, red) and *slc12a1* (DE, green) in WT and heat
1062 shock-treated *hs:tfap2a* embryos at 24 hpf. Scale bar = 35 μm . White box indicates area imaged at higher
1063 (60X) objective in D'. **D'.** DAPI (above) and merged channels (below). Dotted white line outlines a single
1064 cell coexpressing *slc9a3* and *slc12a1* transcripts. DAPI (blue) labels nuclei.

1065

1066

1067 **Figure 5: *tfap2a* is essential for the induction of terminal differentiation, but not cell proliferation,**
1068 **survival, polarity or ciliogenesis within the distal nephron. A.** WISH used to stain *dlx2* (pharyngeal
1069 arches, purple), *slc9a3* (panproximal, purple), and *slc12a3* (DL, red) performed on 24 hpf WT and *trm*^{-/-}

1070 embryos to assess nephron patterning. Black dotted lines indicate presumptive area occupied by DE
1071 progenitors. Scale bars = 70 μ m. **B.** WISH analysis of *dlx2* and renal specification markers that span entire
1072 tubule (*cdh17* and *hnf1ba*) in WT and *trm*^{-/-} at 24 hpf. Green boxes indicate continuous expression of tubule
1073 markers in DE. Scale bar = 70 μ m. **C.** Whole mount FISH and IF to visualize proliferating DE cells
1074 (*slc12a1*, green) in WT and *trm*^{-/-} at 24 hpf. anti-ph3 (red) labels proliferation. White dotted lines outline
1075 pronephric tubule (bottom). Scale bar = 10 μ m. **D.** Acridine orange assay reveals no detectable difference
1076 in dying cell number (green) in WT and *trm*^{-/-} at 24 hpf. White box indicates inset (optical zoom) of distal
1077 nephron area. Scale bar = 70 μ m. **E.** Survey of epithelial polarity proteins by whole mount IF in WT and
1078 *trm*^{-/-} at 4 dpf. anti-Na,K-ATPase (red) was used as a basolateral marker and anti-aPKC (green) was used
1079 as an apical marker. Top image represents WT Na,K-ATPase protein expression in 4 dpf. White boxes
1080 indicate region in E'. Scale bars = 200 μ m, 10 μ m. **E'.** Regions highlighted in B showing normal protein
1081 localization. **F.** Whole mount FISH with IF to assess cilia (anti-acetylated tubulin, green) morphology in the
1082 DE (*slc12a1*, red) of WT and *trm*^{-/-} at 24 hpf. White dotted lines demarcate nephron. Scale bar = 10 μ m.
1083 DAPI (blue) labels nuclei.

1084

1085

1086 **Figure 6: *tfap2a* interplays with the Iroquois homeobox genes *irx3b* and *irx1a* during**
1087 **nephrogenesis. A.** Whole mount double FISH in 20 ss WT animals reveal *tfap2a* transcripts (green) and
1088 *irx3b* transcripts (red) are coexpressed distal pronephros. White box focuses on area of intense
1089 coexpression. Bottom panel indicates area outlined by white box at 60X magnification. DAPI (blue) labels
1090 nuclei. Scale bars = 70 μ m, 10 μ m. **B.** Top panel: WISH for *irx3b* (purple) and *smyh1* (red) in WT and
1091 *tfap2a* morphants at 24 hpf. White dotted line indicates *irx3b* expression domain, which does not change
1092 due to *tfap2a*-deficiency. Bottom panel: WISH for *tfap2a* expression in *irx3b* morphants at 24 hpf. Black
1093 box indicates presence of *tfap2a* transcripts in DE segment domain in WT. Red box indicates absence of
1094 *tfap2a* transcripts in DE segment domain in *irx3b* morphants. Red asterisks (*) indicate loss of *tfap2a*
1095 expression within the neural crest streams in *irx3b* morphants. Scale bar = 70 μ m. **C.** Quantification of
1096 *tfap2a* expression domain length. Measurements were compared by unpaired t-test. Data are represented
1097 as \pm SD. ***p<0.001. **D.** WISH reveals reduced *irx1a* expression in *trm*^{-/-} and *tfap2a* morphants as
1098 compared to WT at 24 hpf. Scale bar = 35 μ m. **E.** WISH of *irx1a* expression in *hs:tfap2a* untreated and
1099 heat shock-treated (red HS+) at 24 hpf. Black dotted lines denotes increased expression of *irx1a* in heat
1100 shock-treated *hs:tfap2a* embryos. Scale bar = 35 μ m. **F.** Quantification of *irx1a* expression domain length in
1101 WT, *hs:tfap2a* (untreated), *trm*^{-/-}, *tfap2a* morphants, and heat shock treated (red HS+) *hs:tfap2a*.
1102 Measurements were compared by ANOVA. Data are represented as \pm SD. **p < 0.01, Green brackets
1103 indicate not statistically significant.

1104

1105

1106 **Figure 7: *tfap2a* and *tfap2b* function in a proposed genetic regulatory network to control distal**
1107 **nephron differentiation. A.** Schematic comparing DE progenitor maturation in WT and *trm*^{-/-}. Mutant cells
1108 display no perturbations in the early specification of the renal lineage (marked by *lhx1a*, *pax2a*, and
1109 *hnf1ba*). Mutant progenitors undergo segment specification and exhibit features of mature epithelium
1110 (*cdh17*, Na,K-ATPase, and acetylated tubulin). In the final phase of differentiation, mutant cells fail to
1111 express DE-specific solute transporters (*slc12a1*, *romk2*, and *clcnk*). B. Diagram depicts *tfap2a* distal
1112 nephron GRN. *irx3b* promotes *tfap2a* expression, and *tfap2a* functions upstream of *irx1a* (green arrows).
1113 *tfap2a* acts upstream of *tfap2b* as the core regulator of solute transporter expression (orange arrows).
1114 *tfap2b* functions redundantly (purple dotted line) to activate distal solute transporters (*romk2*, *slc12a1*,
1115 *clcnk*, and *slc12a3*).

1116

1117

1118

1119

1120 Supplemental Figures & Figure Legends

1121

1122 **S1 Figure: *Tfap2a* amino acid sequence is highly conserved across vertebrate species.** Depicts
1123 amino acid alignment of human, mouse, and zebrafish *Tfap2a* generated by T-coffee and Boxshade online
1124 tools. DNA-binding and dimerization motifs (blue line) display a high degree of sequence similarity (>90
1125 percent). Black boxes mark conserved residues.

1126

1127

1128 **S2 Figure: *tfap2a* MO splice efficacy verification through RT-PCR analysis. A.** *tfap2a* exon map,
1129 indicating *tfap2a* MO-splice (red) targets the 3' end of exon 2. Forward and reverse primers used for RT-
1130 PCR analysis are situated within exon 2 and exon 3 (green arrows). B. Image of RT-PCR gel reveals
1131 presence of a larger product size in the morphant lane, indicating disrupted splicing. 1 = WT band, 2 =
1132 morphant band. C. Table presenting the sequencing results of each band. D. WT and *tfap2a* morphant
1133 amino acid sequences. Inclusion of intron 2-3 results in premature stop codon (red), and a predicted
1134 truncated protein in *tfap2a* morphants.

1135

1136

1137 **S3 Figure: *tfap2b* MO splice efficacy verification through RT-PCR analysis. A.** *tfap2b* exon map,
1138 indicating *tfap2b* MO-splice (red) targets the 3' end of exon 4. Forward and reverse primers used for RT-
1139 PCR analysis are situated within exon 4 and exon 5 (green arrows). B. Image of RT-PCR gel reveals
1140 presence of a larger product size in the morphant lane, indicating disrupted splicing. 1 = WT band, 2 =
1141 morphant band. C. Table presenting the sequencing results of each band. D. WT and *tfap2b* morphant

1142 amino acid sequences. Inclusion of intron 4-5 results in premature stop codon (red), and a predicted
1143 truncated protein in *tfap2b* morphants.

1144

1145

1146 **S4 Figure : *trm*^{-/-} mutant embryos exhibit normal filtration and proximal tubule fluid uptake at 3 dpf.**

1147 **A.** WISH analysis of DE markers (*slc12a1* and *romk2*) in WT and *trm*^{-/-} mutants at 4 dpf. Scale bars = 200
1148 μm, 50 μm. **B.** Kidney function assay was performed by injecting 40 kD Dextran FITC into the circulation of
1149 2 dpf WT and *trm*^{-/-} larvae. Images were collected 1-hour post injection and 24-hours post injection. Right
1150 panel: nephron tubules labeled with green fluorescence indicate endocytosis of Dextran. Scale bar = 150
1151 μm. **C.** O-dianisidine staining of craniofacial vasculature in WT and *trm*^{-/-} at 4 dpf. Abnormal cartilage is
1152 annotated by black arrowhead. Scale bar = 100 μm.

1153

1154

1155 **S5 Figure: *trm*^{-/-} mutant embryos exhibit normal proximal nephron segment pattern formation but**

1156 **have abrogated corpuscle of Stannius formation.** **A.** WISH to evaluate proximal convoluted tubule
1157 (*slc20a1a*) and proximal straight tubule (*trpm7*) in WT and *trm*^{-/-} at 24 hpf. Black arrowheads indicate the
1158 start and end of *slc20a1a* expression. Scale bar = 50 μm. **B.** Quantification of absolute lengths of *slc20a1a*
1159 expression. **C.** Quantification of absolute lengths of *trpm7* expression. n = 3 for each control and test
1160 group. Measurements were compared by unpaired t-test. Data are represented as ± SD. N.S. = not
1161 significant. **D.** WISH to assess corpuscle of Stannius (*stc1*) development in WT and *trm*^{-/-} at 48 hpf. Black
1162 line indicates *stc1* expression domain. Scale bar = 20 μm.

Figure 1

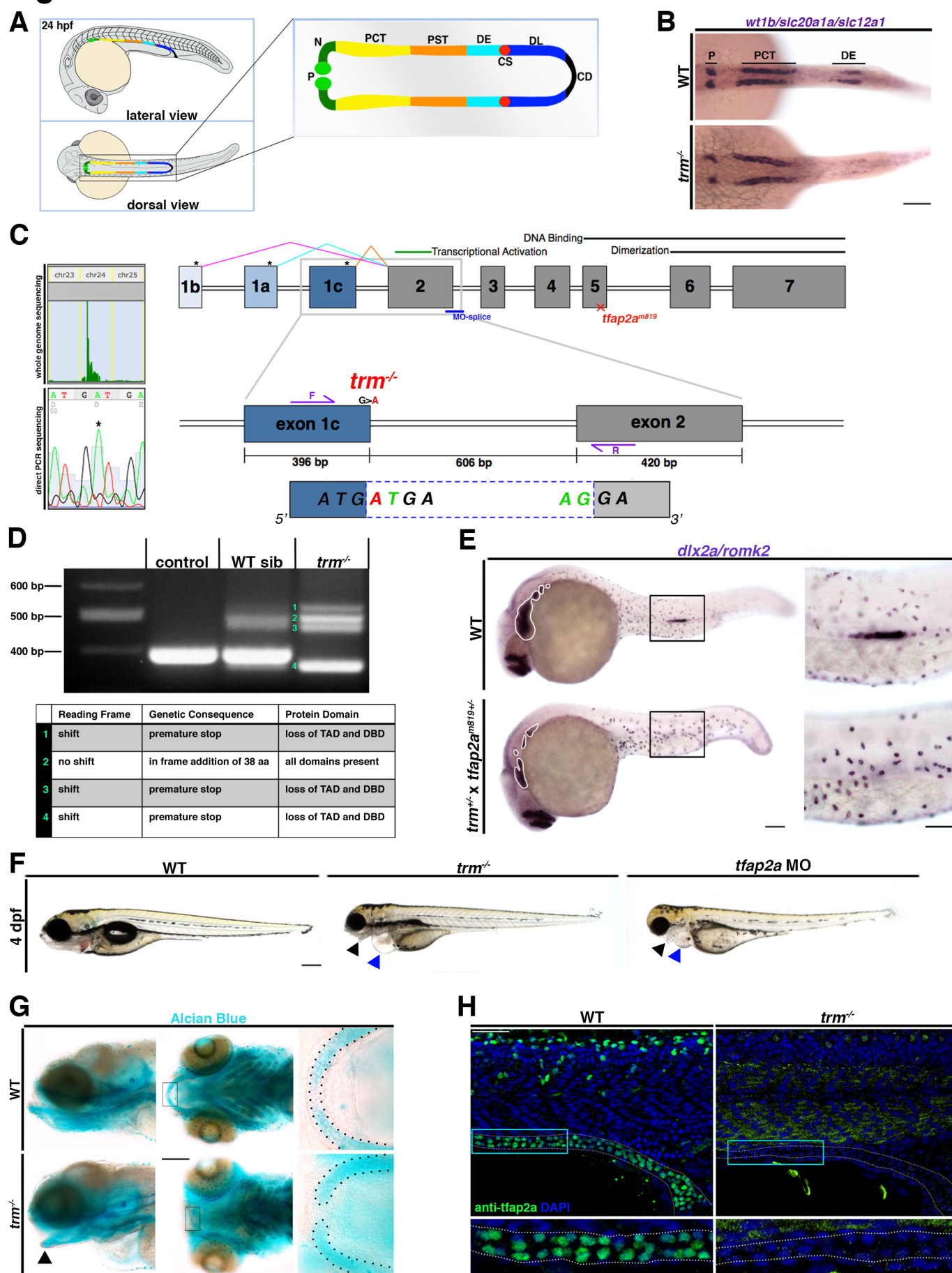


Figure 2

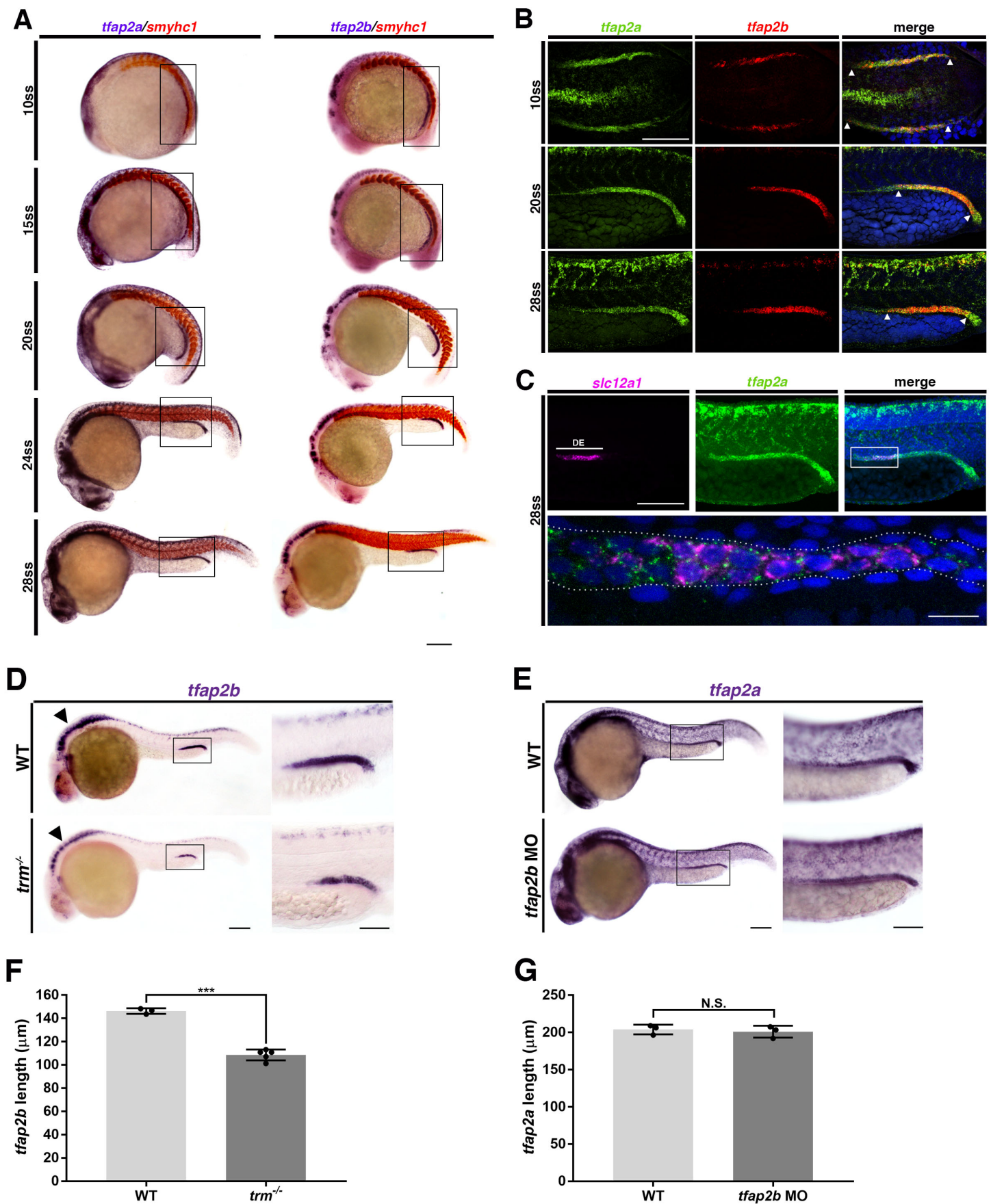


Figure 3

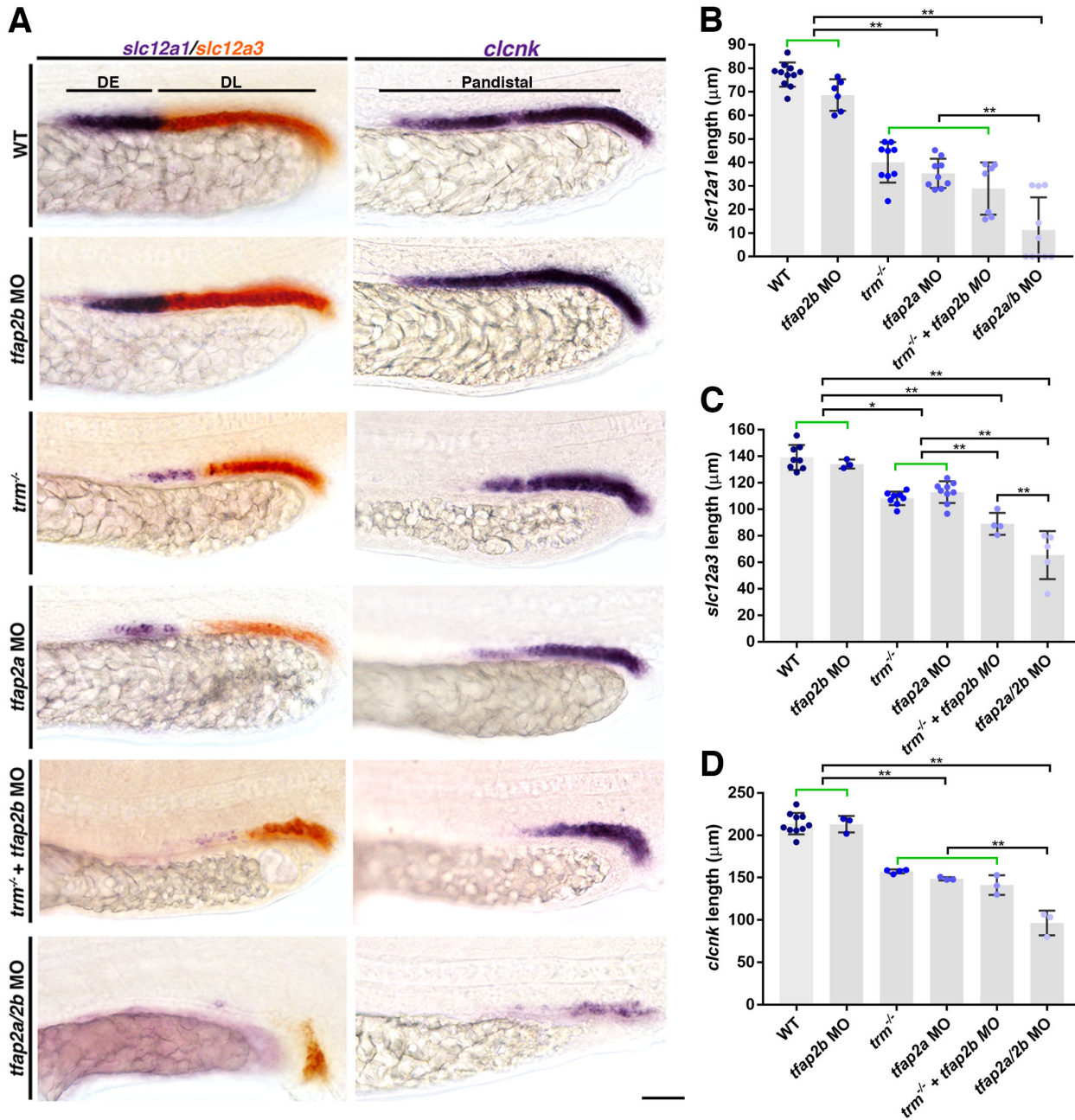


Figure 4

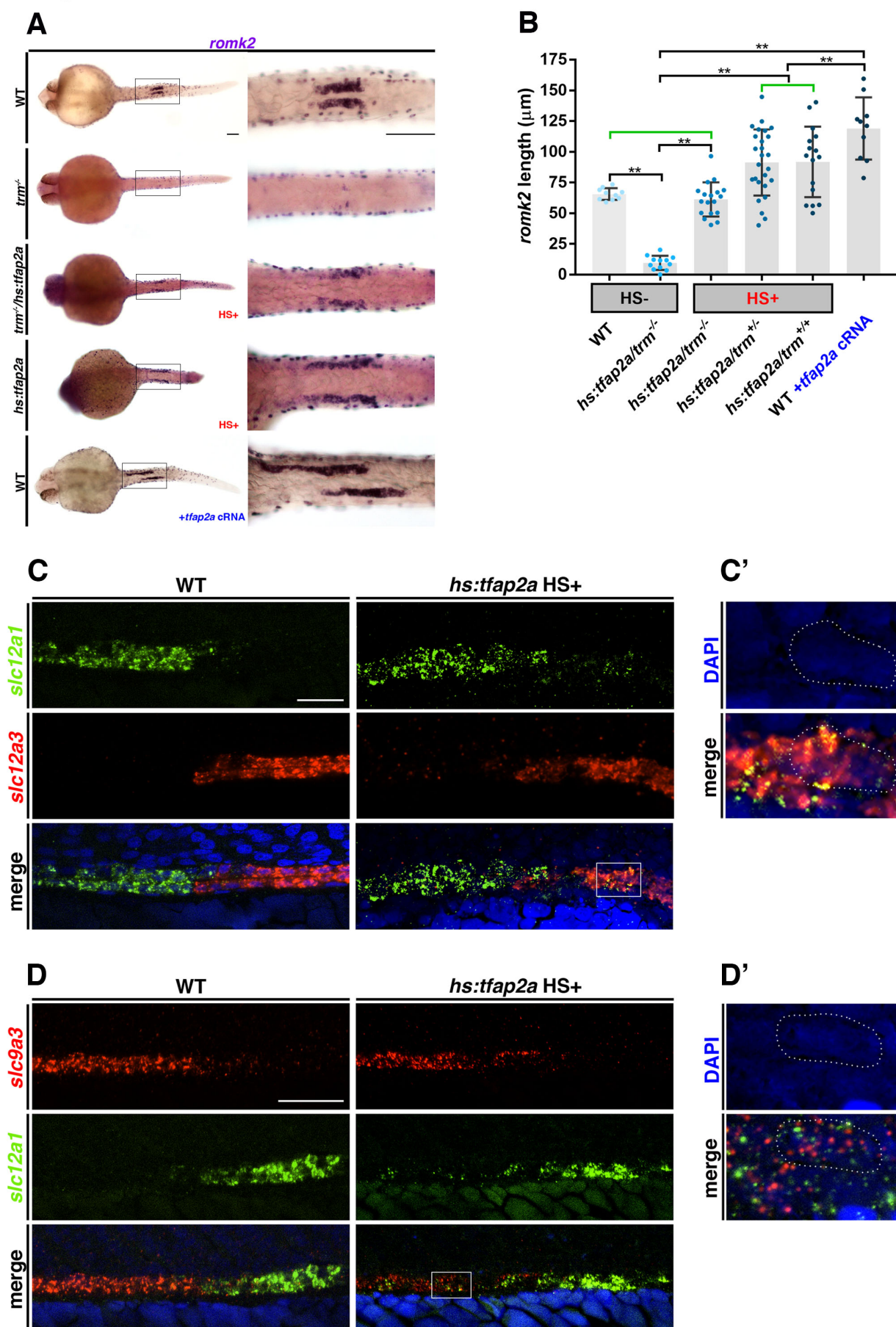


Figure 5

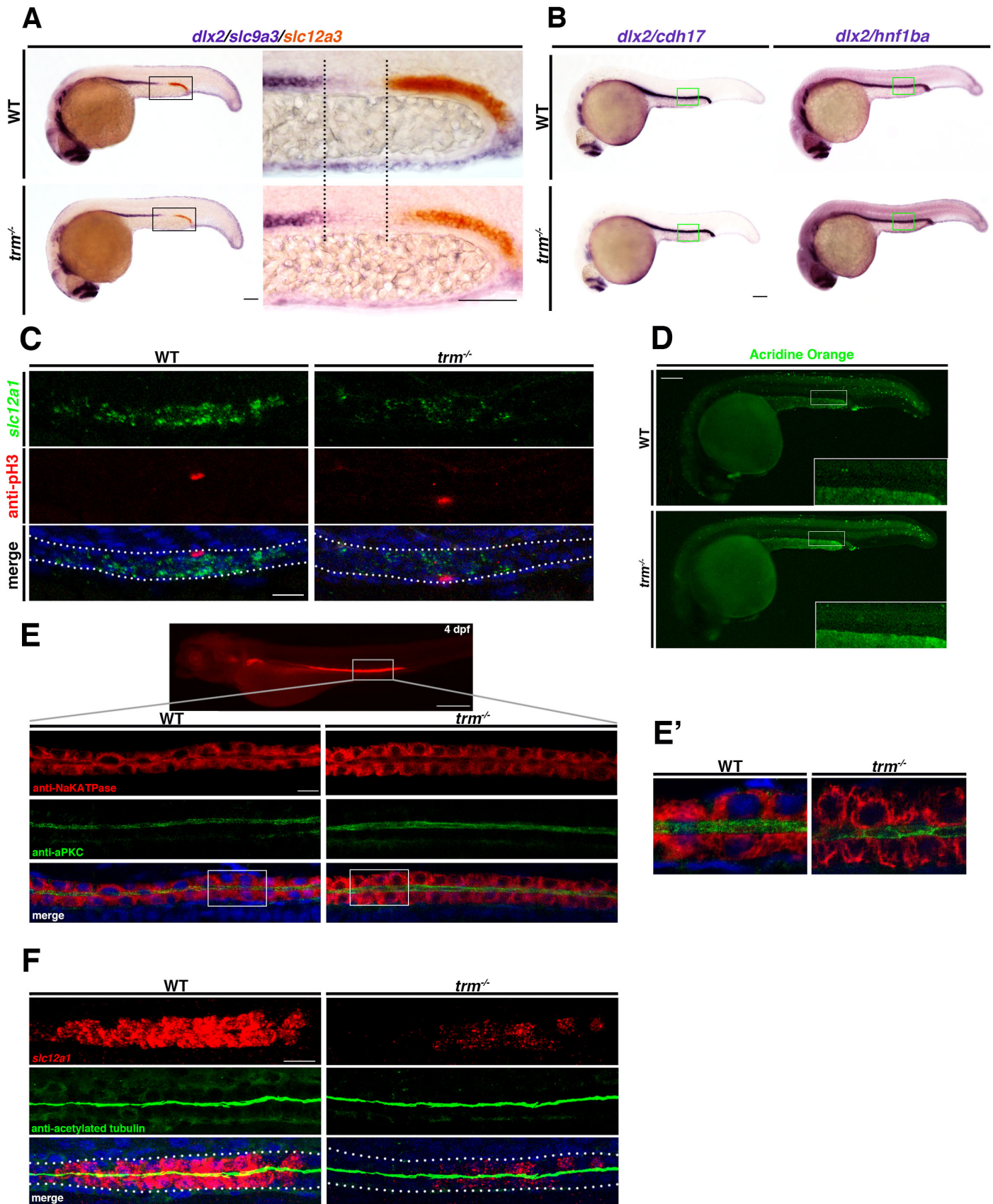


Figure 6

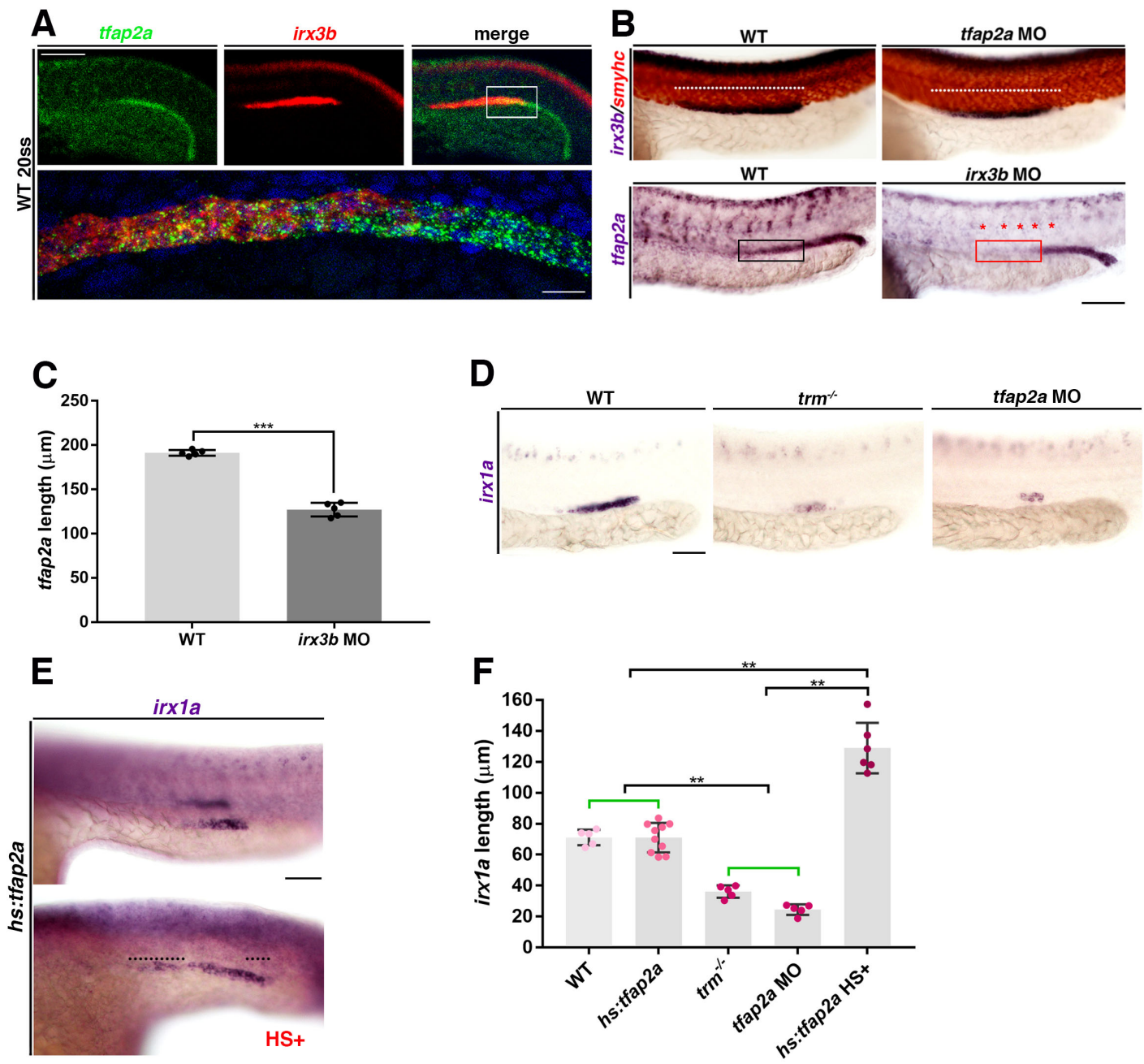
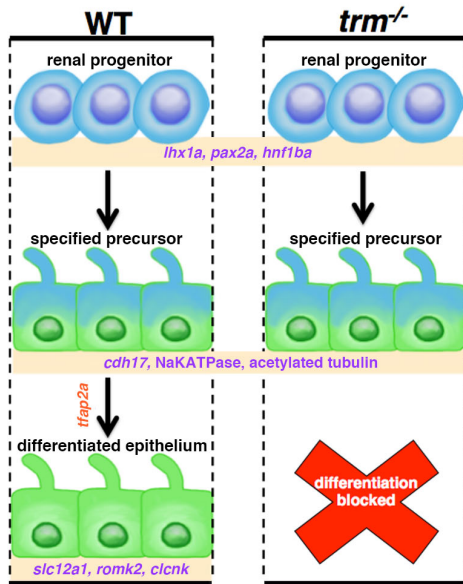


Figure 7

A



B

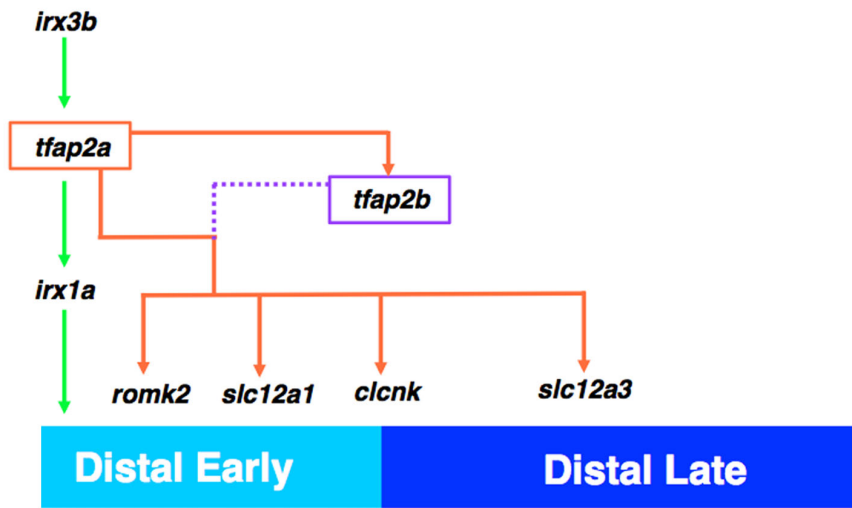


Figure S1

tfap2a protein alignment

```
human      1  -----MLWKLTDNIKYEDCEDRHDGTSNGTARLPQL
mouse     1  MNSVVVDTPFYFGGLPTLGLWNCFSRVVEALRLISSSPSLAEFDLDRHDGTSNGTARLPQL
zebrafish 1  MHCVKIK-----RRRTALYPQPMKMLWKLTDNIKYEDFEDRHDGTSNGTARLPQL

human     32  GTVGQSPYTSAPPLSHTPNADFQPPYFPPPYQPIYQSQDPYSHVNDPYSLNPLHAQPQP
mouse    61  GTVGQSPYTSAPPLSHTPNADFQPPYFPPPYQPIYQSQDPYSHVNDPYSLNPLHAQPQP
zebrafish 51  GSVGQSPYTSAPPLSHTPNNSDFQPPYFPPPYQPIYQSQDPYSHVNDPYSINSLHAQSQP

human     92  QHPGWPGQRQSQESGLLHTRGLPHQLSGLDPRRDYRRHEDLLHGPHALSSGLGDLSTHS
mouse    121  QHPGWPGQRQSQESGLLHTRGLPHQLSGLDPRRDYRRHEDLLHGPHGLGSGLDLPIHS
zebrafish 111  QHPGWPGQRQSQESSLLHQHRLGLPHQLC-----REYRREVLTPSCHGIDTGLTDSIPIHG

human    152  LPHAIEEVPHVVEDPGINIPDQTVIKKGPVSLSKSNSNAVSAIPINKDNLFGGVVNPNEVF
mouse    181  LPHAIEDVPHVEDPGINIPDQTVIKKGPVSLSKSNSNAVSAIPINKDNLFGGVVNPNEVF
zebrafish 166  IPHSLIEDVQQVEDQGIHIPDQTVIKKGPVSIKNNNS-NISAIPIKNDGLFGGVVNPNEVF

human    212  CSVPGRLSLLSSTSKYKVTVAEVQRRLSPPECLNASLLGGVLRRAKSKNGGRSLREKLDK
mouse    241  CSVPGRLSLLSSTSKYKVTVAEVQRRLSPPECLNASLLGGVLRRAKSKNGGRSLREKLDK
zebrafish 225  CSVPGRLSLLSSTSKYKVTVAEVQRRLSPPECLNASLLGGVLRRAKSKNGGRSLREKLDK

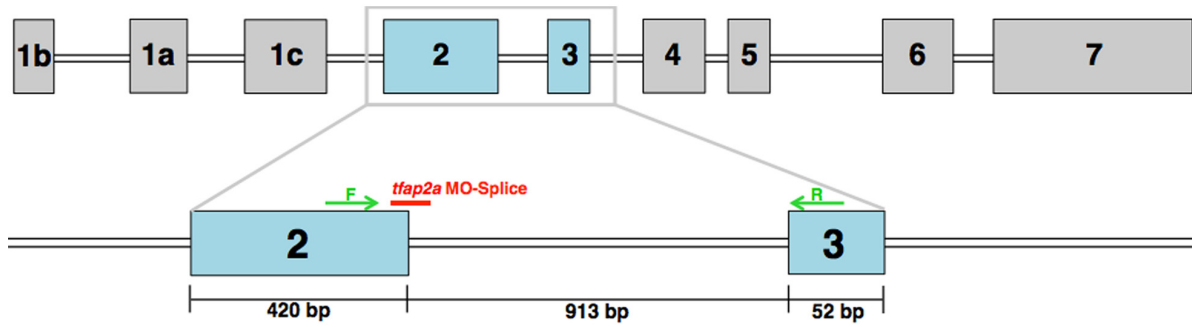
          DNA-binding/Dimerization domain
human    272  IGLNLPAGRRKAANVTLTSLVEGEAVHLARDFGYVCETEFPKAVAEFLNRQHSDPNEQ
mouse    301  IGLNLPAGRRKAANVTLTSLVEGEAVHLARDFGYVCETEFPKAVAEFLNRQHSDPNEQ
zebrafish 285  IGLNLPAGRRKAANVTLTSLVEGEAVHLARDFGYVCETEFPKATAEYMNRRQHSDPNEQ

human    332  VTRKNMLLATKQICKEFTDLLAQDRSPLGNSRPNPILEPGIQSCLTHFNLISHGFGSPAV
mouse    361  VARKNMLLATKQICKEFTDLLAQDRSPLGNSRPNPILEPGIQSCLTHFNLISHGFGSPAV
zebrafish 345  VQRKNMLLATKQICKEFTDLLSQDRSPLGNSRPNPILEPGIQSCLTHFSLISHGFGTIPAV

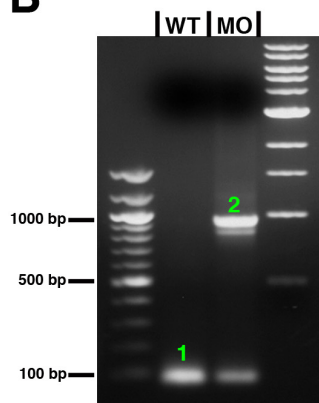
human    392  CAAVTALQNYLTEALKAMDKMYLSNNPNSHTDNNAKSSDKEEKHRK
mouse    421  CAAVTALQNYLTEALKAMDKMYLSNNPNSHTDNSAKSSDKEEKHRK
zebrafish 405  CAAVTALQNYLTEALKAMDKMYLNNNPNSHSETGSKAGDKDEKHRK
```

Figure S2

A



B



C

Band	Size	Result
1	74 bp	WT <i>tfap2a</i>
2	987 bp	Inclusion of intron 2-3

D

WT ...HSLEDVQ QVEDQGIHI...

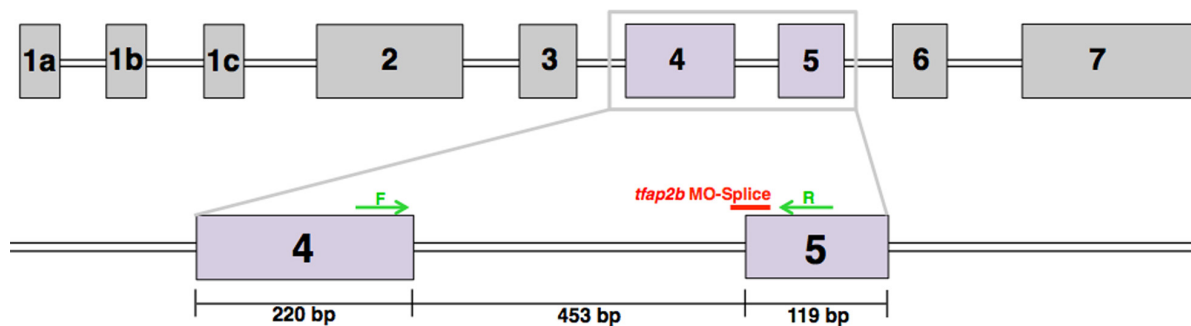
exon 2 exon 3

MO ...HSLEDVQ VRRKAKILQCAAVQRDYSDFLLMH**STOP**

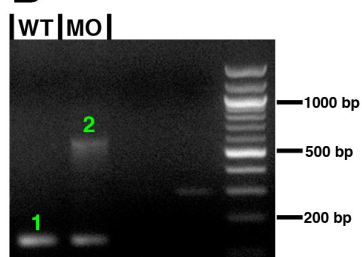
exon 2 intron 2-3

Figure S3

A



B



C

Band	Size	Result
1	135 bp	WT <i>tfap2b</i>
2	588 bp	Inclusion of intron 4-5

D

WT ...CLNASLLGGVLRR AKSKNGGRSLREKL...

exon 4

exon 5

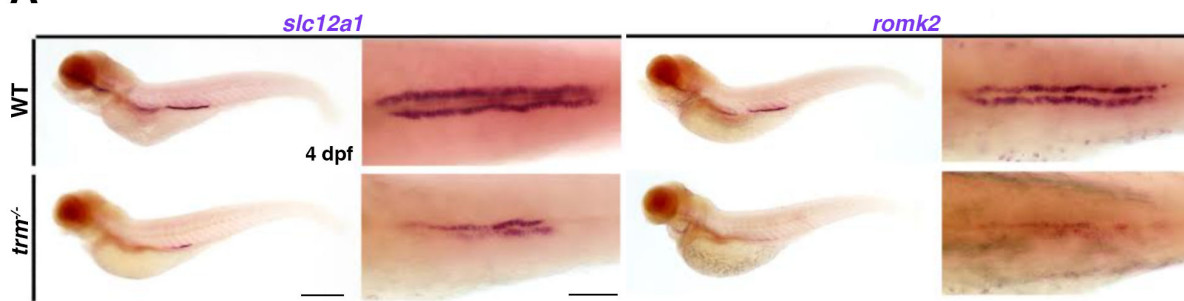
MO ...CLNASLLGGVLRR **STOP**

exon 4

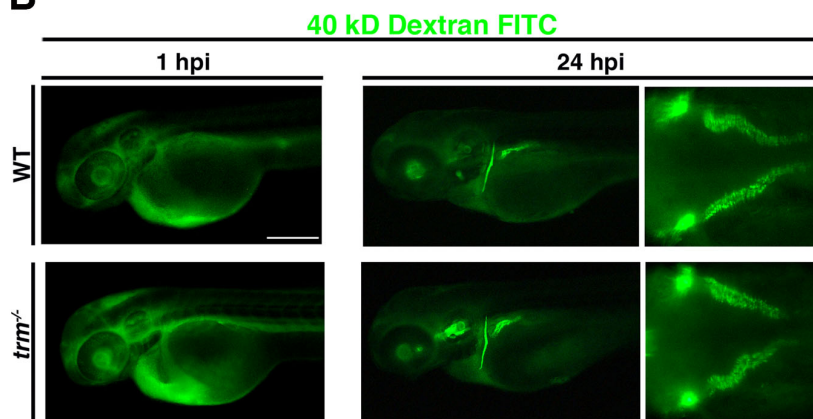
intron 4-5

Figure S4

A



B



C

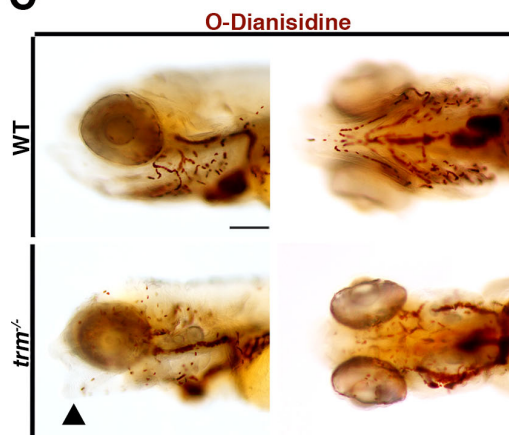


Figure S5

