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Functional studies of a conserved protease-like extracellular matrix protein, Tinagl1, in the zebrafish developmental model

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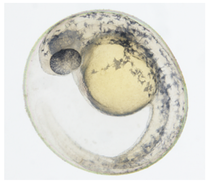


The case FOR Tinagl1 being important to study

- It is a **broadly conserved** extracellular matrix protein, typically found in basement membranes of epithelia and vascular smooth muscle
- Cell culture studies have shown at least three distinct physical and functional interactions with secreted signaling molecules or their receptors

	Effect on signaling	Physical interaction
Wg (fly Wnt)	+	Wg (lipid-dpdt)
Focal adhesion	+ adhesion - FAK kinase	*n.d.* Integrin (blocks ligand binding)
EGF	-	EGF-R (block dimerization)
TGF-β	+	*n.d.*

The case AGAINST Tinagl1 being important to study

- Human disease and trait associations are few/limited (craniosynostosis, metastasis, nephronophthisis [Tinag]; general intelligence)
  - But, observed/expected LOF allele ratio is 0.38 [0.24-0.64] (gnomAD database), indicating selection pressure for function
- A published knockout mouse is viable and fertile
  - But, observed fetal losses across pregnancy weren't characterized
  - Compensation by a related gene, *tinag*, found in mammalian genomes, or other ECM was not studied/ruled out

We are on Team PRO-Tinagl1 – Let's find a better *in vivo* model!

- Zebrafish has only one ortholog, more similar to *tinagl1* than *tinag*
  - Reduces potential for functional compensation
- Of accessible models in our med school environment, zebrafish was the genetic and developmental non-mammalian model organism with organ systems closest in development to mammals
  - PI background is in *Drosophila*, and learning curve, plus funding and health issues, have made this move a challenge

Here we show:

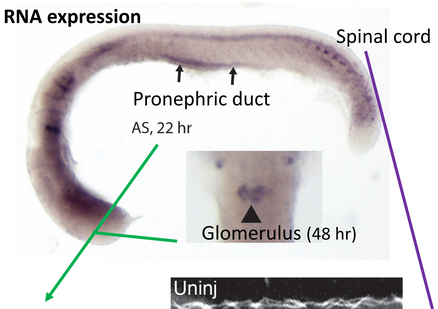
- ➢ Highlights of 2012-2015 work using acute knockdown methods (MOs, crispants; H.N.) that support functional requirements for Tinagl1 in development of pronephros, spinal cord, and heart
  - ➢ Ciliopathy-like?
- ➢ Brief introduction to current approaches being undertaken by PI and undergraduate students
  - ➢ CRISPR knockout for stable loss of function/genetics
  - ➢ Dominant-negative mutants (B.Z.)
  - ➢ Localization of GFP-tagged Tinagl1 (H.B.)

Selected background references: PNAS 96:11323-8, 1999; PLoS ONE 5:e13905, 2010; PNAS 109:370-7, 2012; J Reprod Dev 62:43-9, 2016; Cancer Cell 35:64-80, 2019

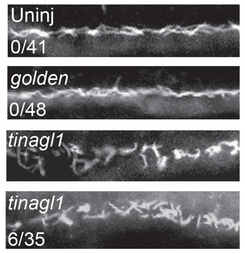
2012-2015 Our initial forays show functional requirements in pronephros and spinal cord, tissues in which *tinagl1* is clearly expressed. Cilia defects *may* underlie at least the defects in pronephros and in heart lateralization, while neuronal and neural crest *may* have adhesion-related defects in migration, survival, and/or proliferaton

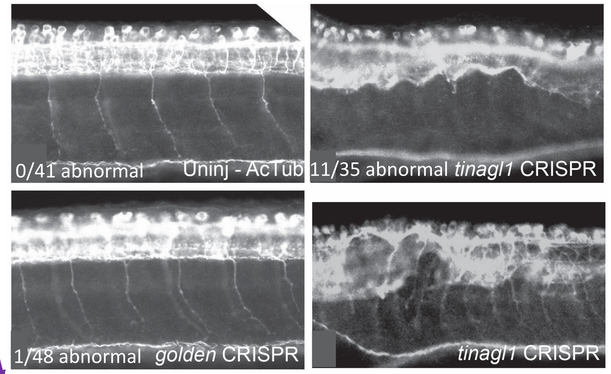
- **Pre-CRISPR – we tested 2 splice-blocking MOs, with mismatch controls and RNA rescue** *(mostly not shown here).* Results are in agreement with:
  - **Early CRISPR – shown are results for single-guide CRISPR in G0 somatic mosaic embryos, using *golden* as a negative control**

***tinagl1* RNA expression**



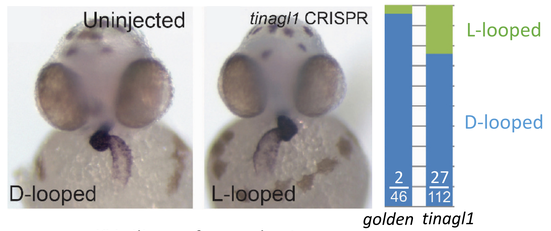
Renal:
- Edema
- Cilia disorganized
- Cilia shorter, fewer (only looked w/MOs)



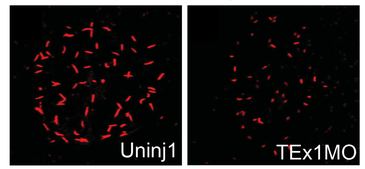


Spinal cord:
- Defects in motor axon outgrowth
- Disorganization of spinal tracts
- Drop-out of sensory neurons

Heart laterality



KV cilia are fewer, shorter



(KV cilia only assessed in MO experiments; other KV parameters not tested)

- ➢ Results on craniofacial defects are published in *Cleft Palate and Craniofacial Journal* (2017) 54: 381-390 (elemosy@augusta.edu to request PDF).
- ➢ I will soon submit the remaining and substantial quantitative MO and G0 CRISPR data as a **hypothesis-generating manuscript** (@DrEllenLeMosy). Acute knockdown methods are non-ideal and cannot be used alone to ascribe function, but may reveal defects hidden by compensation in knockout models.

2019 - ? (COVID hold): Tool-Building To Address Function More Clearly

- Knockout lines **using 2-guide CRISPR to make deletions within key coding sequences** – PI's job, genotyping F1s
- Potential **dominant-negative mutants** that we think will disrupt binding of Wnts – constructs ready to inject as mRNA into early embryos
- **GFP-tagged Tinagl1** for subcellular localization and protein interaction studies - cassette ready to subclone into pTol2 vectors for transgenic expression

▽ CRISPR/Cas9-mediated deletion lines (early truncation or in critical domain)

* Tagged, site-directed mutants for mRNA injection or transgenes

When these tools are in place:
- Evaluate tissue phenotypes and embryo/juvenile development
- Candidate pathway analyses in cilia, ECM, Wnt, and integrin signaling
- Bring in non-biased approaches such as RNA-seq to compare KO to hets and WT

- Your ideas/suggestions/questions welcome here!

Summary and Key Take-Aways

- ➢ Tinagl1 is highly conserved but function *in vivo* is poorly understood
- ➢ Prior studies tie Tinagl1 to multiple signaling pathways, and to processes such as angiogenesis, cancer metastasis, and renal development
- ➢ Our work (recognizing limitations of the knockdown techniques used):
  - ➢ **Renal, heart laterality, and other phenotypes not shown, suggest roles in motile cilia function**
  - ➢ **Spinal cord defects suggest roles in neuronal survival and migration**
  - ➢ Functional analysis will depend on building KO lines

Acknowledgments

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