Photoreceptor Progenitors Depend Upon Coordination of *gdf6a*, *thrβ*, and *tbx2b* to Generate Precise Populations of Cone Photoreceptor Subtypes

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Purpose. Replacing cone photoreceptors, the units of the retina necessary for daytime vision, depends upon the successful production of a full variety of new cones from, for example, stem cells. Using genetic experiments in a model organism with high cone diversity, zebrafish, we map the intersecting effects of cone development factors *gdf6a*, *tbx2b*, and *thrβ*.

Methods. We investigated these genes of interest by using genetic combinations of mutants, gene knockdown, and dominant negative gene expression, and then quantified cone subtype outcomes (which normally develop in tightly regulated ratios).

Results. *Gdf6a* mutants have reduced blue cones and, discovered here, reduced red cones. In combined *gdf6a*/*tbx2b* disruption, the loss of *gdf6a* in heterozygous *tbx2b* mutants reduced UV cones. Intriguingly, when we disrupted *thrβ* in *gdf6a* mutants by using a *thrβ* morpholino, their combined early disruption revealed a lamination phenotype. Disrupting *thrβ* activity via expression of a dominant negative *thrβ* (*dnthrβ*) at either early or late retinal development had differential outcomes on red cones (reduced abundance), versus UV and blue cones (increased abundance). By using *dnthrβ* in *gdf6a* mutants, we revealed that disrupting *thrβ* activity did not change *gdf6a* mutant cone phenotypes.

Conclusions. *Gdf6a* loss directly affects blue and red cones and indirectly affects UV cones by increasing sensitivity to additional disruption, such as reduced *tbx2b*, resulting in fewer UV cones. The effects of *thrβ* change through photoreceptor development, first promoting red cones and restricting UV cones, and later restricting UV and blue cones. The effects of *gdf6a* on UV, blue, and red cone development overlap with, but likely supersede, those of *thrβ*.

Keywords: zebrafish, cone photoreceptor, determination, color vision, regeneration, progenitor, BMP signaling, thyroid signaling, retinal development, retinal lamination

Retinal degenerations are characterized by the gradual death of retinal neurons and become especially debilitating upon the loss of photoreceptors, the neurons that detect light. Cone photoreceptors, or cones, respond to high intensity light and are divided into subtypes based on maximal wavelength sensitivity; thus, they are especially important for daytime visual function and color discrimination. The human macula is populated exclusively by cones and is critical for daytime vision and visual acuity; thus, when retinal degeneration occurs in the macula, functional vision is dramatically compromised. Restoring functional vision in humans demands strategies to replace lost cones, but how cones and their subtypes are produced remains poorly understood. Creating functional cone photoreceptors for therapeutic use (e.g., from stem cells) requires better knowledge and manipulation of the regulatory networks in cone development.[1–10]

Much insight into photoreceptor development to date has come from the mouse, which has two cone types (M and S cones) typical of most mammals. Photoreceptor progenitors are characterized by *Crx* expression; from this pool, *Nrl* and its target *Nr2e3* direct rod photoreceptor fate.[11] Thyroid hormone receptor, or *Thrβ*, is a transcription factor with roles in numerous developing tissues, including the retina, otic vesicle, pituitary, and jaw cartilages.[12–17] In the retina specifically, mouse *Thrβ* and zebrafish *thrβ* are required for M cones and red cones, respectively,[18,19] whereas treatment with thyroid hormone itself initiates death and regeneration of UV cones in trout[20,21] and shifts visual pigment content in zebrafish.[22,23] In mice, it is proposed that *Thrβ* drives the differentiation of M cones, whereas S cones are a default type.[24] RAR-related orphan receptor (ROR) and retinoid X receptor (RXR) nuclear receptors can either negatively (*RXRγ*)[25] or positively (*RORβ*, *RORα*)[26–28] regulate the expression of S-opsin in mice, which results in a dorso-ventral gradient pattern of S-opsin expression. Overall, the mouse retina is dominated by rods with a low density of cones and, thus, a paucity of cone-cone interactions. By contrast, the human macula is cone dominant and has three cone types, more than most mammals. How three (or more) cone subtypes can be created and what conditions are required to build a cone-rich area like the macula remain unanswered and demand a wider diversity of retina development models.

Nonmammalian vertebrates have a greater diversity of cones and so offer insight into how multiple cone subtypes are generated. The zebrafish retina is dense with cones, with four

subtypes based on maximal light spectrum sensitivity (UV, blue, green, and red), and these subtypes are produced in tightly controlled proportions in larvae and adults. Furthermore, these four subtypes are arranged in a precise row mosaic pattern in the adult retina.[29–37] The four major cone types are conserved in the vertebrate lineage, making zebrafish a highly useful model to study cone development. In zebrafish, T-box transcription factor 2b, or *tbx2b*, is a transcription factor required for early neuronal differentiation in the dorsal retina[38] and, specifically, UV cones (the homologue to mammalian S cones and human blue cones); *tbx2b* appears to negatively regulate rod abundance.[39] Growth differentiation factor 6, or GDF6, is a bone morphogenetic protein (BMP) ligand that is known to bind BMP receptors such as BMPR1 and BMPR2, thereby activating SMAD transcription complexes.[40–43] *GDF6* disruption is causal of congenital photoreceptor defects in Leber congenital amaurosis (LCA17).[44] In zebrafish, the homologue to GDF6 *gdf6a* is required for establishing dorsal identity in the early retina,[45–47] for regulating retinal cell proliferation,[48] and for adequate blue cone abundance.[35] *Gdf6a*$^{s327/s327}$ mutants are microphthalmic and have reduced blue cone to UV cone abundance ratios. *Gdf6a* also enhances *tbx2b* for the correct production of UV cones and rods. *Six7*, a *sine oculis* homeobox transcription factor, is required for green cones.[49,50]

In sum, while the murine retina contains two cone types with one developmental transcription factor complex discerning between them,[18,24,51] zebrafish have four cone subtypes produced in exact ratios[29–31,33,52,53] and so must use a more complex system of progenitor fate choice. Much of the knowledge of zebrafish cone subtype production is expected to be directly relevant to the development of the human macula. We sought to determine if the factors within this system interact to regulate the ratios, or relative abundances, of cone subtypes. In this study, we examined *thrβ*, *gdf6a*, and *tbx2b*.

Our documentation of the cone phenotype in *gdf6a* mutants is expanded: *gdf6a* mutants have reduced blue and red cones but not UV cones, suggesting that *gdf6a* promotes blue and red cone development. In addition, the interaction between *gdf6a* and *tbx2b* is newly detailed by quantifying cone abundances, confirming that *gdf6a* loss causes progenitors to have reduced tolerance for insufficient *tbx2b* activity, resulting in fewer cells assuming a UV fate. We find that *thrβ* has developmental stage-specific roles in red cone and UV and blue cone subtype specification and interacts with *gdf6a* to direct proper retina lamination.

Methods

Ethics Statement

All fish care and experiments were approved by the Animal Care and Use Committee: Biosciences at the University of Alberta under protocol AUP00000077 and were in accordance with the Canadian Council on Animal Care. Similarly, the work adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Animal Care and Embryo Injections

Zebrafish were raised and maintained according to standard procedures.[54] Embryos were kept in E3 embryo medium at 28°C except where indicated in the experimental procedures. Medium with 0.003% propylthiouracil to prevent pigmentation was applied at 8 to 10 hours post-fertilization (hpf). Mutant lines were gifted as follows: *gdf6a*$^{+/s327}$ (Zebrafish Information network [ZFIN] identifier [ID] ZDB-ALT-050617-10)[45] from Andrew Waskiewicz (University of Alberta) and *tbx2b*$^{+/fby}$ (ZFIN ID ZDB-ALT-070117-1)[55,56] from Josh Gamse (Vanderbilt University). Transgenic lines used were *Tg(-5.5opn1sw1:EGFP)*^{kj9} (ZDB-ALT-080227) and *Tg(-3.5opn1sw2:mCherry)*^{ua3011} (ZDB-TGCONSTRCT-130819-1).[34,35,57,58] For *thrβ* knock down experiments, embryos were injected with 10 ng of either splice-blocking *thrβ* morpholino (MO) (5′-TCTAGAACTTGCAATACCTTTCTTA-3′) (ZFIN ID ZDB-MRPHLNO-131114-1) (predicted to retain the intron between exons 1 and 2)[19,59] or standard control MO (5′-CCTCTTACCTCAGTTACAATTTATA-3′)[60] at the 1- to 2-cell stage and then were maintained as above.

Generating Transgenic *dnthrβ* Zebrafish

Dominant negative thyroid hormone receptor beta was generated by expressing a version of the cDNA modified to lack 12 amino acids from the carboxy terminus, thereby removing the ligand binding and coactivator binding sites, as previously accomplished in *Xenopus*.[61,62] Primers used in construct creation were based upon *dnthrβ* mRNA of the National Center for Biotechnology Information Reference Sequence NM_131340.1 and are 5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC AGT ATG TCA GAG CAA GCA G-3′ (forward) and 5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG AGC TCT GTG GGA CAT TCC-3′ (reverse). Transgenic constructs were created using multisite Gateway cloning into vectors suitable for Tol2 recombination and transgenesis.[63] The sequence encoding dominant negative thyroid hormone receptor beta, or *dnthrβ*, was amplified from zebrafish cDNA and recombined with the hsp70 promoter sequence (allowing for conditional expression of the dominant negative receptor) and V2A.NLS.eGFP (for visualization of expression) to create the plasmid pDestTol2CG2.hsp70:dnthrβ.V2A.NLS.eGFP. The V2A.NLS.eGFP sequence was kindly provided by Steven Leach.[64] This construct was verified by sequencing and injected with Tol2 mRNA into zebrafish embryos at the 1- to 2-cell stage, and transient transgenic fish were raised to establish a stable transgenic line (line designation ua3113). To visualize the UV and blue cones, *Tg(hsp70:dnthrb.V2A.NLS.eGFP)*^{ua3113} fish were crossed to *Tg(-5.5opn1sw1: EGFP)*^{kj9}; *Tg(-3.5opn1sw2: mCherry)*^{ua3011} carriers.[34,35,57,58]

Heat shock was performed at the ages indicated by placing petri dishes (containing embryos in E3 medium) in a water bath set to 37°C for two hours. Under these conditions, the measured E3 medium temperature reached 34°C. Transgene expression was confirmed through GFP expression visualized 4 to 6 hours following heat shock.

Genotyping for *gdf6a* and *tbx2b* Mutations

Genotyping of adult and larval fish was performed via restriction fragment length polymorphism. Genomic DNA was extracted from fin clips from adults and whole larvae as previously described by Meeker et al.,[65] and regions containing the *gdf6a*^{s327} and *tbx2b*^{fby} loci were PCR amplified by using the primers designed by Gosse and Baier[45] for *gdf6a*^{s327} (forward, 5′-ATGGATGCCTTGAGAGCAGTC-3′; reverse, 5′-CTACCTGCAGCCACACTGTTC-3′) and Snelson et al.[66] for *tbx2b*^{fby} (forward, 5′-TGTGACGAGCACTAATGTCTTCCTC-3′; reverse, 5′-GCAAAAAGCATCGCAGAACG-3′). PCR products underwent restriction digest with BmsI (*gdf6a*) and SaqAI (*tbx2b*) (FastDigest, FD2124 and FD2174) and were run on a 3% agarose gel. The *gdf6a*^{s327} mutation is detected by the loss of a BmsI restriction site, such that the wildtype product is digested into 170- and 110-bp bands, and the mutant product remains a single 280-bp band. The *tbx2b*^{fby} mutation

introduces a novel SaqAI restriction site; the wildtype product runs as a single 206-bp band, and the mutant product results in 169- and 37-bp products.

Immunocytochemistry and Imaging

Larvae were fixed at 4 days post-fertilization (dpf) in 4% paraformaldehyde overnight, and whole-mount immunocytochemistry was performed as previously described.[35,36] Briefly, larvae underwent washes in 0.1 M PO_4/5% sucrose, then in 1% Tween/H_2O (pH 7.4), and then in −20°C acetone. Larvae were incubated with 10% normal goat serum/PBS^{3+} for 60 minutes to reduce nonspecific antibody binding and incubated overnight at 4°C in PBS^{3+} containing 2% normal goat serum (NGS) and antibody. Following incubation in primary antibody, larvae were washed with PBS^{3+} and incubated in secondary antibody overnight at 4°C.

Immunohistochemistry on cryosections was performed as follows: larvae were fixed at 4 dpf in 4% paraformaldehyde overnight and dehydrated step wise in 0.1M PO_4 with increasing concentrations of sucrose. Larvae were then embedded with frozen section compound (catalog no. 95057-838; VWR, Radnor, PA, USA), and the eyes were cryosectioned (10 µm per section) and mounted on Superfrost Plus slides (catalog no. 12-550-15; Fisher Scientific, Waltham, MA, USA). Sections were stored at −80°C until used, where they were rehydrated with PBS with Tween 20 (PBSTw), blocked in 10% NGS/PBSTw for 30 to 90 minutes, and incubated in primary antibodies diluted in 2% NGS/PBSTw overnight at 4°C. Slides were subsequently washed and incubated in secondary antibodies overnight at 4°C. All immunohistochemical steps were performed in a humid chamber.

Monoclonal primary antibodies and dilutions used were mouse 1D4 against zebrafish red cones (1:500; ZFIN ID: ZDB-ATB-110114-2; catalog no. b5417; Abcam, Cambridge, MA, USA) and rat 10C9.1 against UV opsin (1:100; ZFIN ID: ZDB-ATB-140728-2).[35] Secondary antibodies/stains and dilutions used were Alexafluor 647 chicken anti-mouse (1:1000; catalog no. A-21463; Invitrogen, Carlsbad, CA, USA), Alexafluor 488 chicken anti-rat (1:1000; catalog no. A-21470; Invitrogen), and 4′,6-diamidino-2-phenylindole (catalog no. D1306; Invitrogen). Retinas were then dissected and flat mounted. Imaging of flat-mount and sectioned tissues was performed using a Zeiss LSM 700 scanning confocal microscope and Zen 2010 software (Carl Zeiss, Oberkochen, Germany).

Image Analysis and Statistical Analysis

Images of flat-mounted retinas were analyzed in ImageJ (https://imagej.nih.gov/ij/index.html, Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). In each retina image, cone subtypes were counted within a 100 µm × 100-µm area (drawn as a square dorsal of the optic nerve head). For *gdf6a* mutants, most flat-mounted retinas were amenable to counts in a 100 µm × 100-µm area, so they were used for analysis. Raw values of cone subtype counts are available in the Supplementary Figures. Cone subtype abundance data were analyzed via Kruskal-Wallis tests with Mann-Whitney pairwise comparisons in Stata/SE 14.1 for Mac (2015, StataCorp, College Station, TX, USA). Sample sizes (n) reported in the figures represent the number of larval fish examined.

Results

Gdf6a Influences *tbx2b* in UV Cone Development

We had previously investigated the interaction of *gdf6a* and *tbx2b* in photoreceptor development by using the $tbx2b^{lor}$ and $tbx2b^{fby}$ mutants (hypomorphic and null alleles, respectively). $Tbx2b^{lor/lor}$ mutants display the "lots-of-rods" phenotype with an overabundance of rods and a small number of UV cones, and $tbx2b^{fby/fby}$ mutants have lots of rods and a near-complete absence of UV cones.[39] Although zebrafish heterozygous for $tbx2b^{lor}$ or $tbx2b^{fby}$ have typical rod and UV cone abundances, in a $gdf6a^{s327/s327}$ background (often abbreviated here as $gdf6a^{-/-}$), some $tbx2b^{+/lor}$ or $tbx2b^{+/fby}$ embryos have an overabundance of rods.[35] Here, we reexamined the effects of this interaction and expanded upon it by assessing abundances of UV, blue, and red cones.

Quantification of cone abundances confirmed phenotypes that have been documented previously, including homozygous $tbx2b^{fby/fby}$ mutants that lack UV cones[39] and $gdf6a^{s327/s327}$ mutants that have a reduction in blue cones (39% fewer than wildtype, $P = 0.003$). Additionally, we found that $gdf6a^{s327/s327}$ mutant retinas have a novel phenotype of fewer red cones (29% reduction, $P = 5.16 \times 10^{-5}$) (Fig. 1; abundances are shown as ratios to control values, which are normalized to 1, and average values of raw counts are available in Supplementary Fig. S1). In our previous publication, only the UV and blue cones were examined and quantified,[35] so this red cone phenotype had not been appreciated. Cone abundances of fish heterozygous for either $gdf6a^{s327}$ or $tbx2b^{fby}$ were not different from wildtypes (Fig. 1).

Fish heterozygous for $tbx2b^{fby}$ but lacking *gdf6a* ($gdf6a^{s327/s327}$; $tbx2b^{+/fby}$) had a 23% reduction in UV cone abundance compared to wild types, a partial but statistically significant reduction ($P = 0.014$) (Fig. 1). For $gdf6a^{s327/s327}$ mutants, blue cones were reduced further with the $tbx2b^{fby}$ mutation (81% reduction, $P = 8.16 \times 10^{-5}$ in $gdf6a^{s327/s327}$; $tbx2b^{+/fby}$ and 63% reduction, $P = 6.49 \times 10^{-6}$ in $gdf6a^{s327/s327}$; $tbx2b^{fby/fby}$). Red cone abundance remained low in all $gdf6a^{s327/s327}$ retinas, but interestingly, this abundance was reduced further with sequential accumulation of *tbx2b* mutant alleles (51% reduction, $P = 8.16 \times 10^{-5}$ in $gdf6a^{s327/s327}$; $tbx2b^{+/fby}$ and 64% reduction, $P = 2.37 \times 10^{-6}$ in $gdf6a^{s327/s327}$; $tbx2b^{fby/fby}$).

Thrβ Knock Down in *gdf6a* Mutants Disrupts Cone Abundances and Retinal Lamination

Based on our cone quantifications in *gdf6a* mutants, Gdf6a signaling positively influences red cone and blue cone development. Although the loss of *gdf6a* alone does not alter UV cone abundances, it appears to increase sensitivity toward UV cone loss, as seen in *tbx2b* heterozygous mutants. *Thrβ* is required for red cone specification, but it also negatively regulates UV cones, as *thrβ* morphants have a UV cone excess.[19] Therefore, *gdf6a* and *thrβ* have similar effects on red cone populations but differing effects on UV cone populations.

We wanted to resolve if *gdf6a* and *thrβ* act in shared or separate regulatory pathways toward UV cone fate. We generated two hypotheses: (1) *gdf6a* and *thrβ* act independently in UV cone development, where Gdf6a signaling promotes UV cones indirectly (possibly linked by *tbx2b*), and Thrβ suppresses UV cones separately from that; or (2) UV fate is regulated by *gdf6a* and *thrβ* in an epistatic fashion. For hypothesis 1, we predicted that reduction in both factors, and reduction of their opposing influences, would result in a wildtype abundance of UV cones. For hypothesis 2, we predicted that the reduction of both would cause either excess of UV cones (if *thrβ* knock down can override any sensitization to UV cone reduction as caused by *gdf6a* loss) or a reduced abundance of UV cones (if *thrβ* knock down does not rescue sensitization). To test these predictions, we knocked down *thrβ* in $gdf6a^{s327/s327}$ mutants with a *thrβ* splice-blocking morpholino, which was previously used by others.[19]

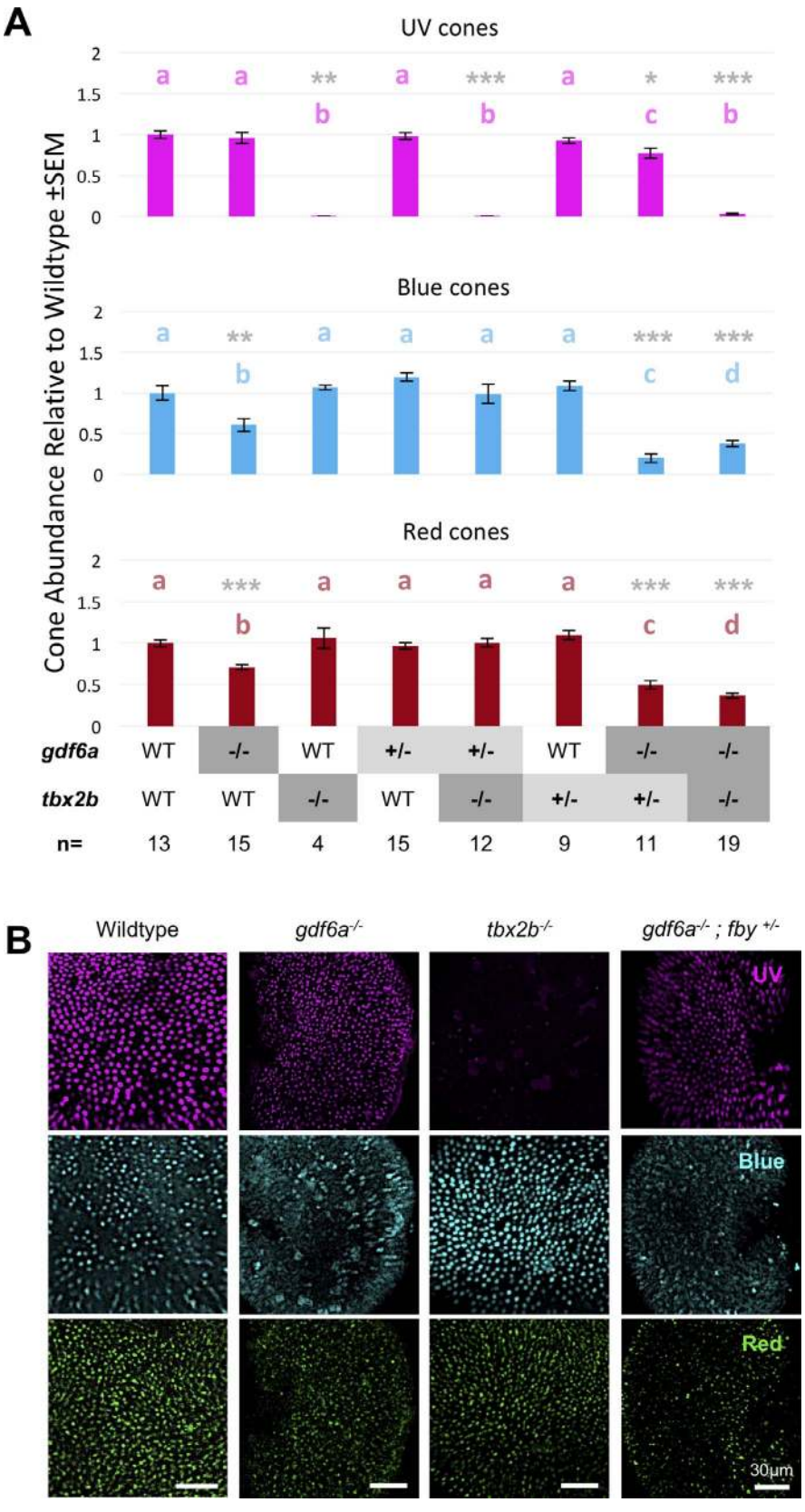

Figure 1. *gdf6a* loss in *tbx2b* heterozygous *fby* carriers reduces UV cone abundance. (**A**) Relative abundances of UV, blue, and red cones in compound $gdf6a^{+/s327}$; $tbx2b^{+/fby}$ in-crosses show near-complete loss of UV cones in $tbx2b^{fby/fby}$ mutants and partial loss of UV cones in $tbx2b^{+/fby}$ mutants with $gdf6a^{s327/s327}$ background (labeled $gdf6a^{-/-}$ or "–/–"). Values with matching letters are not significantly different (Kruskal-Wallis test with Mann-Whitney pairwise comparisons, *gray asterisks* indicate $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ relative to wild type (WT)/WT controls). $Gdf6a^{s327/s327}$ mutants have reduced blue cones and a novel reduced red cone abundance phenotype, both of which appear either unaffected or slightly enhanced by *tbx2b* loss. Wild-type values shown are sibling controls. (**B**) Representative retinal images depicting UV (*magenta*), blue (*cyan*), and red cones (*green*) in wild type, $gdf6a^{s327/s327}$, $tbx2b^{fby/fby}$, and compound $gdf6a^{s327/s327}$; $tbx2b^{+/fby}$ mutants.

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Disruption of *thrβ* expression with 10 ng of morpholino in *gdf6a*$^{s327/s327}$ mutants yielded a surprising set of results. When we examined whole-mount retinas with antibody-labeled UV and red cones, *thrβ* morphants showed a consistent lack of red cone labeling regardless of *gdf6a* genotype, assuring us of the morpholino's efficacy (91% and 97% decrease in wild type and *gdf6a*$^{s327/s327}$, respectively; $P = 3.64 \times 10^{-5}$, $P = 5.53 \times 10^{-5}$, respectively). In *gdf6a*$^{s327/s327}$ mutant retinas receiving *thrβ* morpholino, the low blue cone phenotype persisted and, in fact, dramatically worsened (98% decrease, $P = 0.00005$). In the UV cone channel, gaps or holes in the photoreceptor layer were apparent in these *gdf6a*$^{s327/s327}$; *thrβ* morphants, which affected UV cone abundance counts (Fig. 2A, 2B; average values of raw counts available in Supplementary Fig. S2). Radial sections of 4dpf *gdf6a*$^{s327/s327}$; *thrβ* morphant retinas revealed lamination defects impacting all retinal layers. Gaps in the photoreceptor layer were confirmed, and "bridges" of cells were found spanning the inner plexiform layer between the inner nuclear layer and ganglion cell layer (Fig. 2C). These lamination defects were not detectable when either *thrβ* or *gdf6a* expression were disrupted individually. This lamination phenotype suggests *thrβ* and *gdf6a* may be active in an early retinal development process, such that the loss of one or the other has no overt effect, but the loss of both negatively affects photoreceptor populations and disrupts lamination.

Differential Disruption of *thrβ* Reveals New Roles in Cone Development

The severity of the lamination defect limited our ability to quantify cone subtypes when *gdf6a* and *thrβ* expression were both disrupted, so, to overcome this, we created a model of conditional Thrβ activity disruption via expression of a dominant negative receptor. It was determined through morpholino knock down that *thrβ* is required for red cone development[19]; however, morpholinos must be introduced at the 1- to 2-cell stage, prohibiting manipulation of *thrβ* expression at later developmental stages. To further investigate *gdf6a* and *thrβ*, as well as actions of *thrβ* at different stages of cone photoreceptor development, we created a transgenic line with a construct created with Gateway recombination[63] that enables the expression of a dominant negative receptor predicted to disrupt endogenous receptor activity. Modeling after the work of Ulisse et al.[67] expressing a dominant negative *thrβ* in *Xenopus*, we amplified the zebrafish *thrβ* sequence and removed nucleotide bases coding for the C-terminal 12 amino acids (which are necessary for ligand and coactivator binding). Without these binding sites, the receptor may dimerize with other receptors and bind to DNA target sequences but fail to recruit the rest of the activation complex and so transcription is not initiated. Expression of this dominant negative receptor (*dnthrβ* for short) was placed under the *hsp70* promoter to enable conditional induction upon heat shock and is followed by enhanced green fluorescent protein (eGFP) (Fig. 3A). This construct was injected into 1- to 2-cell stage embryos and a stable *Tg(hsp70:dnthrβ1V2A.eGFP)* line was established (designation ua3113). Heat shock induction allows us to temporally manipulate *dnthrβ* expression, thereby disrupting the activity of endogenous Thrβ proteins at specific developmental stages. It is important to note that the presence of a dominant negative receptor stands in contrast to MO knockdown (in which overall receptor protein abundance is reduced), as it is predicted to create an incomplete complex that occupies promoter and enhancer regions to the exclusion of other complexes; therefore, it is predicted to have negative transcriptional effects.

Upon heat shock, we observed zebrafish embryos showing eGFP expression throughout the body (Fig. 3B). Without heat shock induction, eGFP expression is limited to the eye lens, which expresses *hsp70* during normal development.[68]

We examined the effects of *dnthrβ* expression on cone photoreceptor subtype determination in comparison to splice-blocking morpholino knockdown of *thrβ*.[19] Knockdown of *thrβ* causes near-complete absence of red cones and an increase in UV cone abundance (by approximately 35%, $P = 0.0003$), whereas expression of *dnthrβ* via heat shock at 52 hpf leads to increased UV (by 27%, $P = 0.003$) and blue cone abundance (by 36%, $P = 0.001$) relative to heat shocked nontransgenic siblings (Fig. 3C; average values of raw counts available in Supplementary Fig. S3). Inducing *dnthrβ* expression at other time points, including 24 hpf, 30 hpf, and 36 hpf, did not alter cone abundances as dramatically relative to controls (<20% change, Supplemental Fig. S4). None of these interventions with *dnthrβ* expression caused UV, blue, or red opsin coexpression, which was verified in the orthogonal views in both Zen 2010 and ImageJ software. This revealed an effect of *thrβ* that is limited to later photoreceptor development: the endogenous receptor negatively regulates blue cone determination, and the dominant negative receptor would disinhibit this process, allowing for more progenitors to assume a blue fate late in development. In contrast, disrupting Thrβ activity either early (with morpholino knock down) or late leads to more UV cones. Finally, unlike the observed coexpression of red opsin with other opsins upon *thrβ* misexpression,[19] expression of *dnthrβ* did not cause opsin coexpression. The methods used to label cone subtypes for analysis have limited capacity to detect coexpression of a diversity of opsin types; the transgenic expression of GFP in UV cones and mCherry in blue cones is robust, but the fluorescent proteins are restricted to the cell bodies, so visualization of the outer segments was not possible. Only two antibodies, anti-UV opsin and anti-red opsin, were available and used here, and so only coexpression of these opsins would be possible to detect. Thus, although we could not see opsin coexpression, we cannot rule it out as a possibility, especially in experiments where the total number of cones detected appears larger than normal (e.g., in *dnthrβ* retinas, total abundances shown in Supplementary Figures).

Disrupting the effects of Thrβ via dnThrβ expression increased blue cone abundance (Fig. 3C, 3D), making *thrβ* only the second gene identified as affecting blue cone abundances in zebrafish (or any animal), after *gdf6a*.[35] Additionally, similar to morpholino knock down, the *dnthrβ* model has an excess UV cone phenotype; therefore, we used this line to test whether Gdf6a signaling and Thrβ are epistatic in their regulation of UV cone (and/or blue cone) development. With the conditional activation of *dnthrβ* expression, we bypassed the *gdf6a/thrβ* lamination phenotype by disrupting Thrβ activity after lamination. We heat shocked *gdf6a*$^{s327/s327}$; *hsp70:dnthrβ* embryos at 52 hpf, as done previously. But instead of increasing UV and blue cone abundances, expression of dnThrβ in *gdf6a*$^{s327/s327}$ mutants yielded slightly reduced UV and significantly reduced blue populations (by 62%, $P = 1.2 \times 10^{-4}$); thus, *dnthrβ* failed to rescue the *gdf6a* blue cone phenotype. *Gdf6a*$^{s327/s327}$ retinas also retained the phenotype of fewer red cones (up to 35% reduction compared to wildtype, $P = 0.0001$) regardless of *dnthrβ* expression (Fig. 4; average values of raw counts available in Supplementary Fig. S1). No opsin coexpression was detected in mutant retinas with *dnthrβ* expression. Thus,

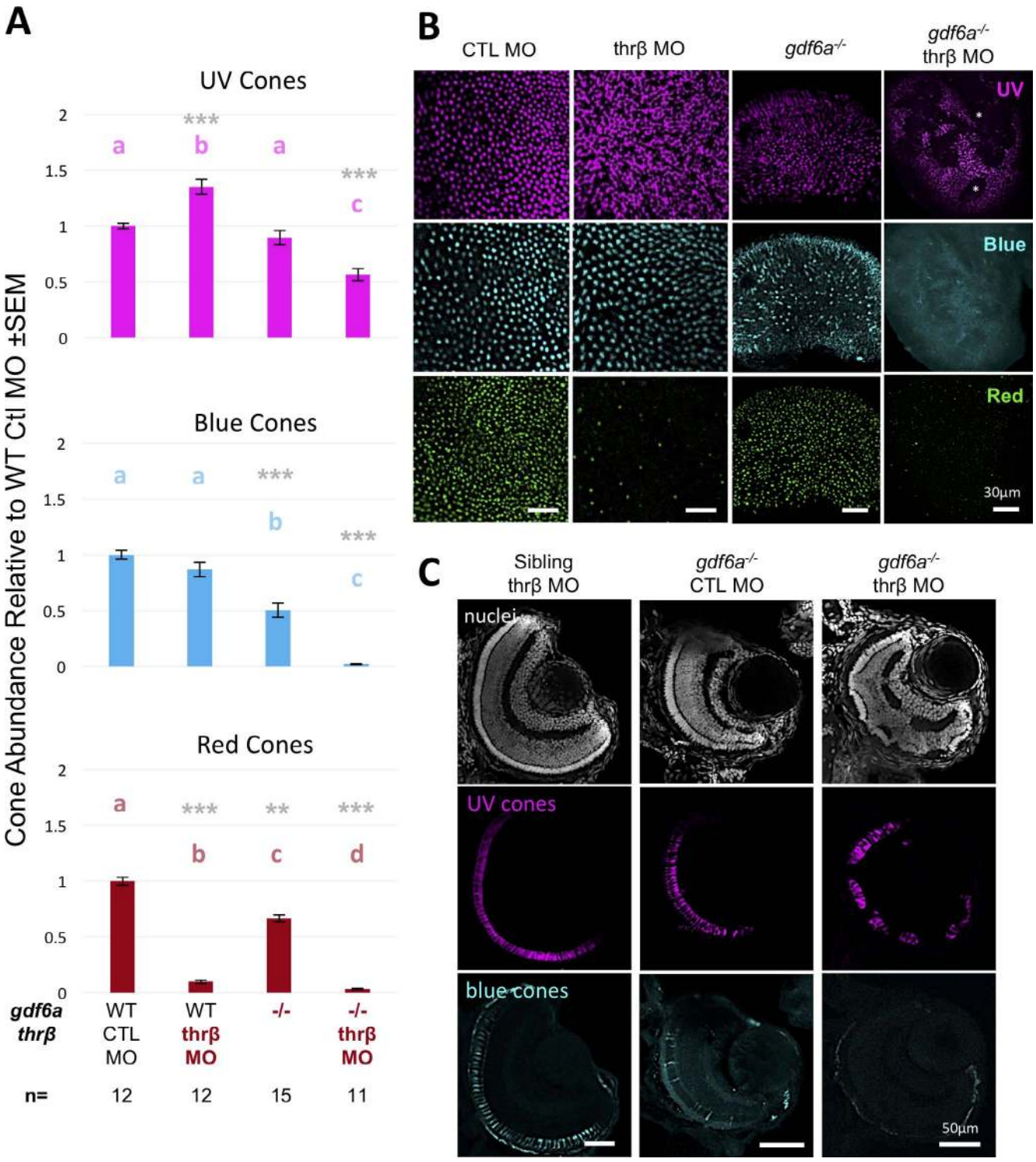

Figure 2. Knockdown of *thrβ* disrupts retinal lamination, including the photoreceptor layer, in *gdf6a*$^{s327/s327}$ mutants. (**A**) *Thrβ* knockdown with splice-blocking morpholino in *gdf6a*$^{s327/s327}$ mutants (labeled *gdf6a*$^{-/-}$ or "–/–") fails to increase UV cone abundance, instead causing near-total loss of blue and red cones. Values with matching letters are not significantly different (Kruskal-Wallis test with Mann-Whitney pairwise comparisons, *gray asterisks* indicate $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ relative to WT + control [CTL] MO controls). UV cone abundance decreased significantly, in contrast to *thrβ* knockdown in wildtype fish. (**B**) Gaps or "holes" can be seen in the photoreceptor layer of *gdf6a*$^{s327/s327}$; *thrβ* MO-treated whole-mount retinas (two holes indicated by *asterisks* in UV image). These holes are not seen in CTL MO-injected *gdf6a*^{s327s327} mutants, nor in wild-type or heterozygous morphants. (**C**) Radial sections of 4 dpf *gdf6a*$^{s327/s327}$; *thrβ* MO-treated retinas show disrupted retinal lamination, with gaps in the photoreceptor layer that are occupied by cells of the inner nuclear layer, and cells disrupting the inner plexiform layer between the inner nuclear layer and ganglion cell layer ($n = 9$ embryos examined per group).

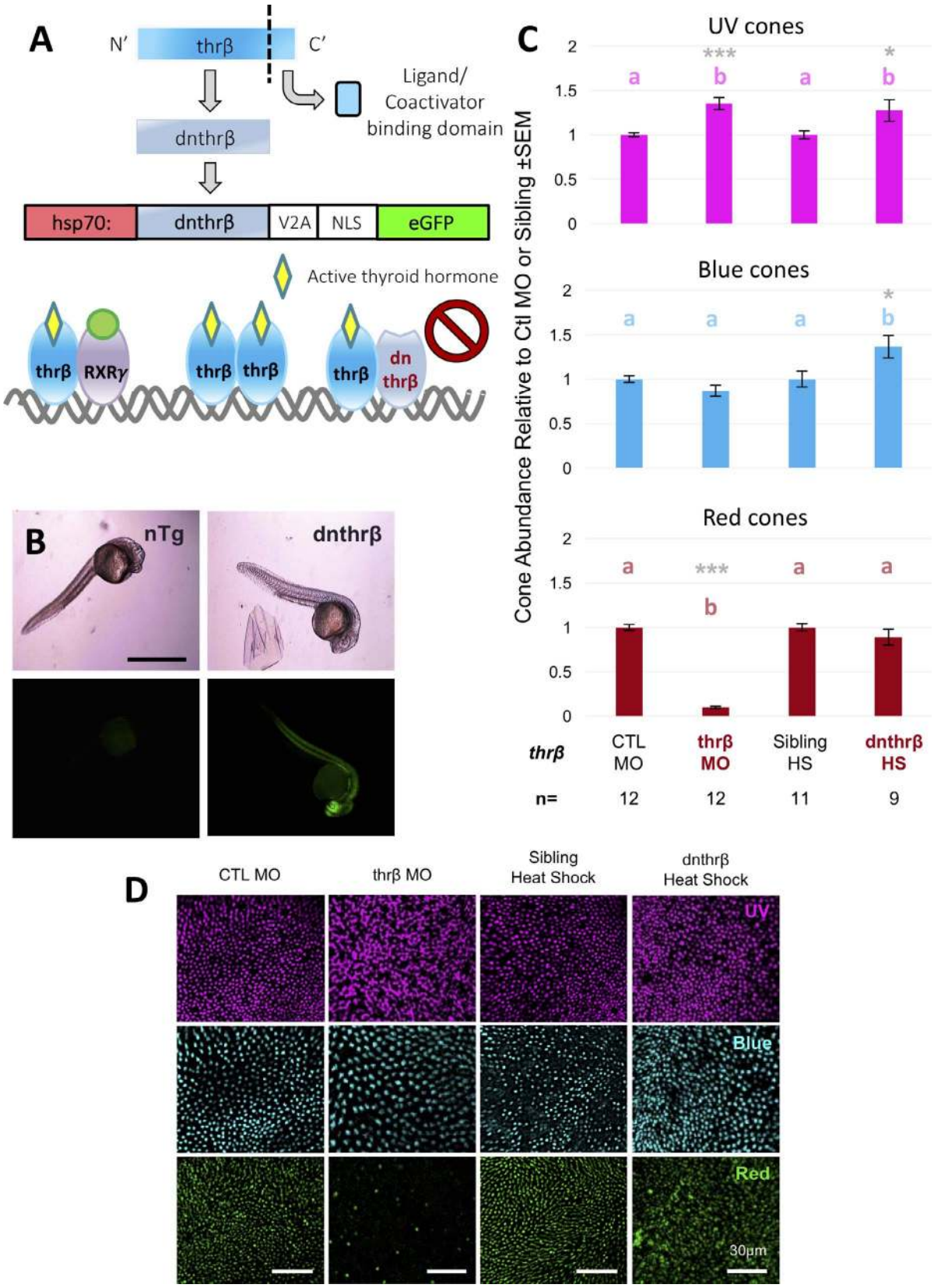

Figure 3. A transgenic zebrafish model of conditional Thrβ disruption reveals additional roles in cone development. (**A**) Generation of a dominant negative thyroid hormone receptor β was accomplished via an 11-amino acid C-terminal deletion in *thrβ*, which was then cloned into a transgene for dominant negative *thrβ* (*dnthrβ*) and eGFP under the hsp70 promoter. Endogenous Thrβ dimerizes with other factors, such as Thrβ or RXRγ, and binds thyroid hormone to activate transcription (*left*), whereas dnThrβ would bind endogenous receptors but, lacking ligand and coactivator binding domains, would render dimers inactive (*right*). (**B**) Transgenic embryos express the transgene, including GFP, throughout the body. *Scale bar*: 1 mm. (**C**) *Thrβ* MO causes dramatic reduction in red cone abundance and increased UV abundance at 10-ng dose. Our dominant negative model shows the same increase in UV cones but also a significant increase in blue cones (HS, heat shocked). Values with matching letters are not significantly different (Kruskal-Wallis test with Mann-Whitney pairwise comparisons, *gray asterisks* indicate $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ relative to CTL MO or sibling heat-shocked [HS] controls). *Thrβ* MO data are normalized to CTL MO data; *dnthrβ* HS data are normalized to sibling HS data.

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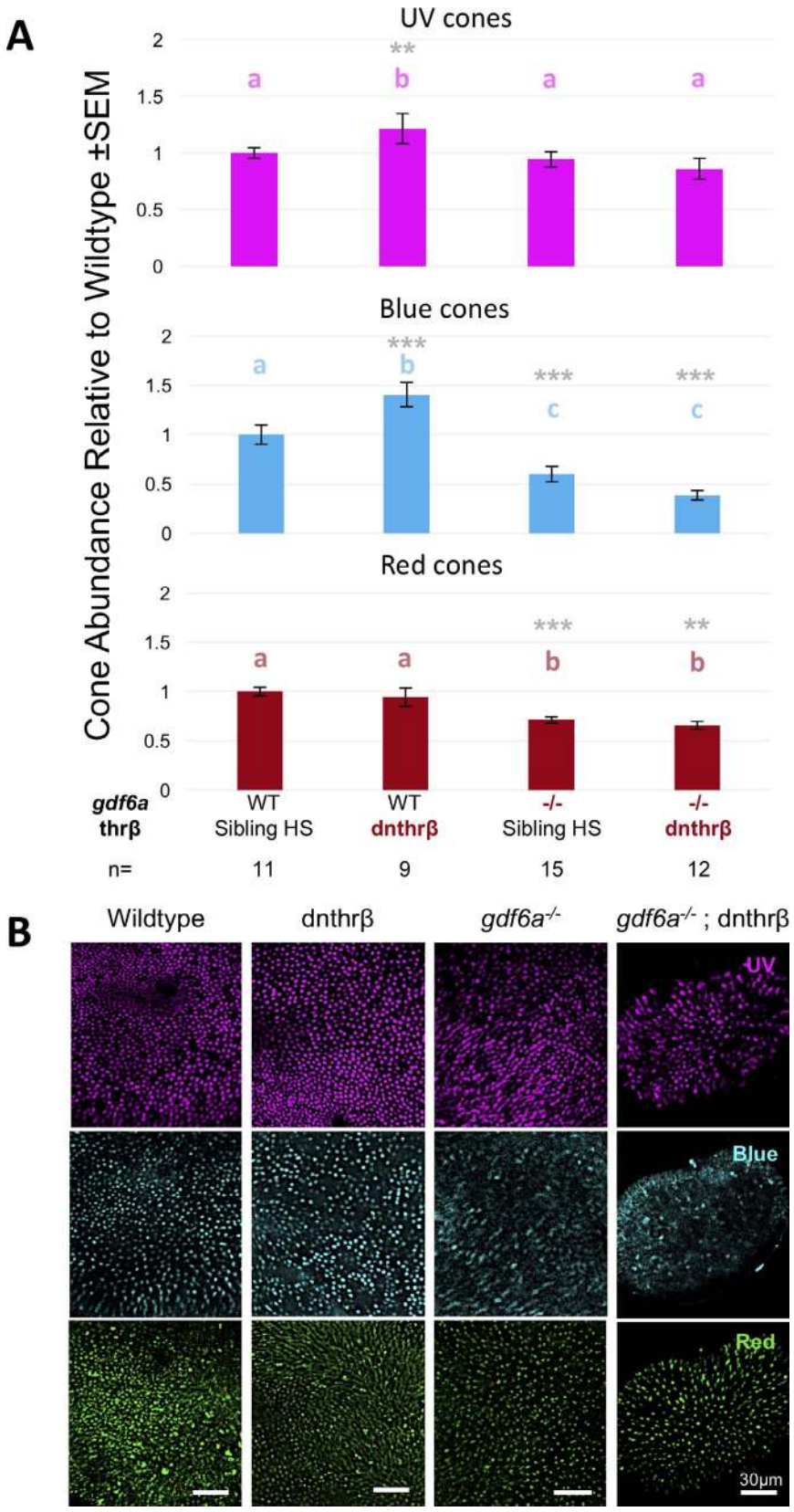

Figure 4. Thrβ disruption with dnThrβ does not rescue the *gdf6*$^{s327/s327}$ blue cone phenotype. (**A**) Expression of dominant negative *thrβ* (*dnthrβ*) increases UV and blue cone abundances, but in *gdf6a*$^{s327/s327}$ mutants (labeled *gdf6a*$^{-/-}$ or "–/–") the low blue cone abundance is not changed and UV cones are not increased with *dnthrβ* expression. *Gdf6a*$^{s327/s327}$ mutants have reduced red cone abundance regardless of *dnthrβ* expression compared to nontransgenic wildtype siblings. Values with matching letters are not significantly different (Kruskal-Wallis test with Mann-Whitney pairwise comparisons, *gray asterisks* indicate $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ relative to WT sibling HS control). (**B**) Representative retinal images of wild-type sibling *gdf6a*$^{s327/s327}$ and *gdf6a*$^{s327/s327}$ with *dnthrβ* expression.

it appears that *thrβ* is not directly downstream of *gdf6a* in blue or red cone fate.

Discussion

In this work, we describe novel genetic roles among *gdf6a*, *tbx2b*, and *thrβ* that impact cone photoreceptor subtype determination, and which elaborate the cone determination regulatory network. We summarize the effects of *gdf6a*, *thrβ*, and *tbx2b* on cone development as found in our experiments here, and then explore the larger implications, including intersecting effects, of these factors on cone subtype development below.

Gdf6a Signaling Positively Regulates *tbx2b* in UV Cone Development

Tbx2b is a downstream target of *gdf6a* in the early retina, contributing to dorsal identity and determination of dorsal retinal cells, and to UV cone development, where *gdf6a* also influences *tbx2b*.[35,45,47,69,70] *Tbx2b* is required for UV cone identity during photoreceptor development; homozygous *tbx2b* mutant retinas have few or no UV cones (*lor* and *fby* alleles are thought to be hypomorphic and null alleles, respectively).[39] These mutants also present with an overabundance of rods (termed the lots-of-rods phenotype).[39] The lots-of-rods phenotype can be elicited in some $tbx2b^{+/lor}$ heterozygous mutants if *gdf6a* is absent (i.e., $gdf6a^{s327/s327}$).[35] Here, we crossed $tbx2b^{fby}$ mutants to $gdf6a^{s327}$ mutants to quantify the effects of the combined mutations on cone subtypes more comprehensively. A 23% reduction in UV cone abundance was observed in the $gdf6a^{s327/s327}$; $tbx2b^{+/fby}$ genotype compared to wild types, and this aligns with previous findings that these compound mutants are predisposed to excess rod generation.[35,39] Thus, *gdf6a* loss in $tbx2b^{+/-}$ mutants likely enhances the sensitivity of progenitors to insufficient levels of *tbx2b* expression, which affects both UV cone and rod abundance (perhaps with some degree of stochasticity, as not all $gdf6a^{-/-}$; $tbx2b^{+/-}$ retinas show reduced UV cones and excess rods). The absence of a lots-of-rods phenotype in *gdf6a* mutants alone suggests that *tbx2b* expression and/or function can be sufficiently supported by factors independent from *gdf6a*, so long as two functional *tbx2b* alleles are present. Thus, the epistatic relationship in UV cone development wherein *tbx2b* would be downstream of *gdf6a* is not a closed system, and other factors may participate in regulating *tbx2b* in this pathway. Reduction in *tbx2b* wild-type gene expression dosage may also worsen *gdf6a* homozygous mutants' blue and red cone phenotypes, although the relevance of *tbx2b* to these cone types is less clear. In summary, *gdf6a* impacts upon both UV and blue cone development, plausibly via stimulating or supporting *tbx2b* expression for the generation of UV cones and possibly via a separate pathway for blue cones (Fig. 5).

Thrβ Promotion of Red Cones and Suppression of UV and Blue Cones Depend on Photoreceptor Development Stage

Thrβ is known to be required for M-opsin and for long-wavelength-sensitive cone identity in mice and in zebrafish[18,19]; this represents a rare shared node in photoreceptor development between these species, and few other such commonalities have been experimentally demonstrated to date (i.e., beyond commonalities in gene expression). Thus, *thrβ* is of deep interest for comparative retinal development, and we sought to extend the characterization of its role and position in the zebrafish photoreceptor regulatory network. With a

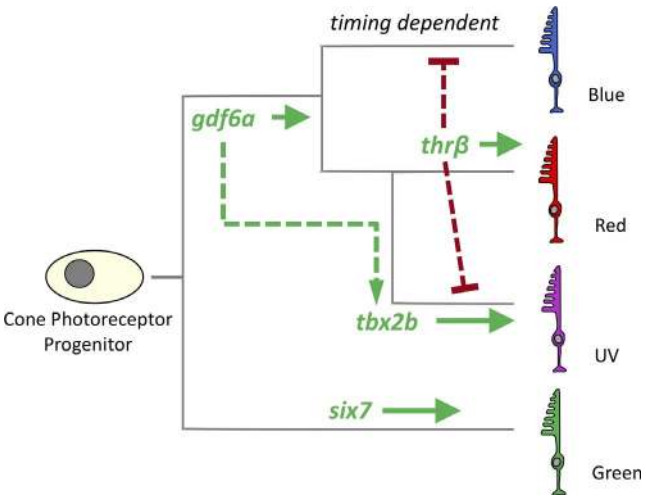

Figure 5. Regulatory actions of *thrβ*, *gdf6a*, and *tbx2b* in zebrafish cone photoreceptor determination. The proposed pathway shown is a summary of the effects of the factors studied on cone subtype development and does not represent order of expression, order of progenitor/precursor progression, or a chronologic sequence of events. *Gdf6a* promotes blue and red cones and indirectly influences UV cones (*dashed arrow*), and *tbx2b* promotes UV cones. *Thrβ* stimulates red cone identity and inhibits UV cones and blue cones (*red dashed lines*). *Gdf6a*'s regulation of blue and red cone abundances supersedes *thrβ*; thus, the effects of *gdf6a* and *thrβ* should be considered independent in the pathway depicted. Work by other groups established that *six7* is required for green cones, but it is not known whether the other factors, such as *gdf6a*, actively regulate green cones.

dnthrβ conditional line, we were able to discern that *thrβ* influences cone progenitors differently depending on developmental age. UV and blue cone abundances were both enhanced, whereas red cones were unaffected when *dnthrβ* was expressed late in photoreceptor development. Thus, we discovered that *thrβ* can negatively regulate zebrafish blue cone fate (mice, like all eutherian mammals, lack this *sws2* cone subtype), in addition to regulating red cones and UV cones.

Reduced *thrβ* expression early in photoreceptor development (via MO knockdown) strongly affects red cone abundance (reduction) and UV cones (increase) but not blue cones. These differential and age-dependent responses to reduced Thrβ (in either expression or activity) suggest that the competency of cone progenitors regarding *thrβ* may change during retinal development. This interpretation of our data is consistent with observations of differential cone abundances when *thrβ* was ectopically expressed under various promoters with differential timing of expression.[19] Red cones appear to have a critical window of induction by *thrβ* shortly after initiation of *crx* expression because in *thrβ* morphants the red cones can be rescued by *thrβ* expression driven by the *crx* promoter (i.e., as early as 19 hpf), as described by Suzuki et al.[19] In the same article, induction of *thrβ* after the final cell division resulted in cones that coexpress red cone opsin with another cone opsin, an imperfect "rescue".[19] Thus, during the critical period for red cone determination, Thrβ induces red cone fate and suppresses UV cone fate, whereas after this period (in postmitotic photoreceptors), Thrβ seems to merely activate red cone opsin expression (and not change cone identity). Likewise, disrupting endogenous Thrβ activity with dnThrβ late in photoreceptor development, as done here, did not change the size of the red cone population. The *dnthrβ* expression line we created did not replicate the reduction of

red cones observed in morphants, which may be due to one or more factors: expression levels of *dnthrβ* may not be sufficient to bind a large enough proportion of endogenous Thrβ proteins to effect measurable change in gene transcription during the critical window for red fate; the critical window for red cone fate was missed; or transcription feedback mechanisms in response to dnThrβ dampened its effects, possibly in part due to the ubiquitous expression of the *dnthrβ* transgene (e.g., enhanced expression of endogenous *thrβ* or use of alternative binding partners to facilitate transcription).

Using our model of conditional disruption of the Thrβ protein, we determined that Thrβ's effect on blue cones is limited to late photoreceptor development. We speculate that *thrβ*'s time-dependent effects on blue cone abundances may be a result of negative transcriptional actions because this phenotype is not observed when *thrβ* expression is merely reduced by MO (see Fig. 2). Thrβ proteins are known to bind and occupy regulatory elements without activating transcription, thereby preventing or reducing gene expression,[71–73] unless (or until) the requisite cofactors are present in adequate amounts, including active thyroid hormone T_3 and coactivators. Thus, Thrβ may limit the number of cone progenitors assuming certain fates (UV cones and blue cones later in development) and promote others (such as red cones) at the appropriate time (Fig. 5). Modification of Thrβ's actions may occur through differential expression of corepressors, such as silencing mediator for retinoid or thyroid hormone receptors (SMRT) and/or dimerization with other thyroid receptors versus retinoid X receptors. As Thrβ is known to interact with RXRs and RORs in mouse development, and zebrafish cone differentiation initiates in the ventral retina where retinoic acid signaling is active,[71,74–78] these interactions are plausible.

Interestingly, inhibition of Thrβ in the adult mouse retina enhances cone photoreceptor survival,[79] which stands in contrast to its necessity for M-cone development and function in mice[80] and zebrafish[19] and possibly similar roles in chicks[81,82] and *Xenopus*[83–85] (although more studies are needed). Thus, the role of *thrβ* is context dependent: *thrβ* promotes specifically red cone identity within a specific window of development; outside this window and outside of red cones, *thrβ* may have negative effects on cone development and survival.

Actions of *thrβ* in Cone Development Are Superseded by *gdf6a*

We next examined *gdf6a* and *thrβ*, as both are implicated in UV cone development. By knocking down *thrβ* in *gdf6a* mutants, we encountered a surprising disruption to lamination across all three retinal layers, as well as near-total lack of blue or red cones.

To resolve whether *gdf6a* and *thrβ* are linked in terms of cone development and to further understand the temporal nature of *thrβ* in cone subtype fate determination, we created a transgenic zebrafish with inducible dominant negative *thrβ* expression to disrupt endogenous Thrβ protein activity.

We then returned to *gdf6a* and *thrβ* with the expanded question of whether they act in shared pathways toward UV or blue cone fate. Our new observation above that *thrβ* is required for suppressing blue cones is noteworthy in contrast to the requirement of *gdf6a* in promoting blue cone development. We, thus, expected to identify epistatic or other intersections between these factors by using combinatorial genetic disruption. Intriguingly, neither the *thrβ* MO nor our *dnthrβ* transgene changed the *gdf6a* phenotype of low blue and low red cone abundances. Although *thrβ* disruption in wild-type retinas increased UV cone abundance, UV cones were not increased in *gdf6a* mutants with *thrβ* disruption. Thus, disrupting *thrβ* failed to rescue blue or red cones or enhance UV cones, suggesting that the influence of *gdf6a* overrides that of *thrβ* in cone subtype determination. In other words, the data do not support that *thrβ* would be directly downstream of *gdf6a* in a shared genetic pathway. Alternative explanations are that *thrβ* and *gdf6a* act in parallel pathways, *thrβ* acts upstream of *gdf6a*, or the early effects of Gdf6a signaling (or lack thereof) on photoreceptor progenitors influences (or constrains) how gene expression may be affected by Thrβ or dnThrβ. For example, *gdf6a* mutant retinas exhibit decreased proliferation and increased apoptosis at 2 dpf.[69,86] The reduction or loss of progenitors, some of which are presumed photoreceptor progenitors, may limit the potential for *dnthrβ* to increase or rescue cone subtype populations. These speculations await further testing. For simplicity, the effects of *gdf6a* and *thrβ* on cone subtype fates are depicted as independent influences in Figure 5.

Gdf6a$^{s327/s327}$ mutants exhibit numerous retinal phenotypes: increased apoptosis[44,69]; a smaller progenitor pool,[69] reduced markers of proliferation, and reduced blue and red cone abundances yet normal UV cone abundances.[35] We speculate that these phenotypes may be interrelated and that the *gdf6a* cone phenotypes may reflect imbalances in progenitor proliferation or survival. Progenitor survival may be impacted further upon decreased *thrβ* expression, resulting in dramatic reductions in differentiated cells, including cones (yet only one cone type appears to persist—UV cones). We speculate the areas lacking photoreceptors in *gdf6a*$^{s327/s327}$; *thrβ* morphants may be artifacts of photoreceptor progenitors that received sufficient doses of *thrβ* MO to cease dividing or to die entirely. Comparing the blue and red cone phenotypes seen in *gdf6a* mutants with either *thrβ* knock down or *dnthrβ* expression, it appears that disruption to their gene functions negatively affects both types. Although *Thrβ* knockdown in *gdf6a* mutants caused photoreceptor loss in parts of the retina, the remaining intact areas contained UV cones but almost no red cones or blue cones (Fig. 2B). Thus, the blue and red cone populations were more severely affected than UV cones. In *gdf6a* mutants expressing *dnthrβ*, the blue and red cone abundances were reduced in an additive fashion as well, although not as dramatically (Fig. 4). These observations suggest that both *gdf6a* and *thrβ* are required to support photoreceptor progenitors (and perhaps progenitors of specific cone subtypes) and to promote red cones. For blue cones, the relationship is less clear: knock down of *thrβ* alone does not affect blue cones but it does in combination with *gdf6a* loss.

Conclusions

The cone photoreceptor development relevant genes *thrβ*, *gdf6a*, and *tbx2b* interact in a fashion that is critical for the production of three cone types, and our work has found that their influences in cone subtype development intersect in intriguing ways, thereby adding new aspects to a model of vertebrate photoreceptor development. This and future work may provide new avenues to explore how the development of color vision may be modified to suit the multitude of visual environments, including diverse color environments, in which vertebrates live.[87] Furthermore, a detailed model of vertebrate photoreceptor development, especially one featuring multiple cone subtypes, is a valuable framework for regenerative medicine. This framework, once established, will guide the modification of existing stem cell protocols and other therapeutic strategies toward higher cone production for restoring daytime vision.

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