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CHARACTERIZATION OF PTH TYPE-2 RECEPTOR (PTH2RB) AND ITS ENDOGENOUS LIGAND PTH2 IN ZEBRAFISH DEVELOPMENT

NICHOLAS SEITZ

72 Pages

Members of the parathyroid hormone family of ligands, PTH (parathyroid hormone), PTH2, and PTHLH (parathyroid hormone-like hormone). These hormones interact with the class B GCPRs parathyroid hormone receptors PTH1R, PTH2R, and PTH1Rb/3R to regulate a variety of biological processes. In mammals, PTH and PTHLH activate PTH1R and PTH1Rb/3R to mediate serum calcium homeostasis, chondrogenesis, and bone maintenance. PTH2R activation by PTH2 has been studied primarily in regard to its neuroendocrine roles but is also found in the skin where it contributes to wound healing and keratinocyte differentiation. Still, little is known about the impacts of PTH2R signaling relative to PTH1R and PTH1Rb/3R. Here, we identify and characterize a paralog of the zebrafish *pth2r* gene we call *pth2rb*. Expression studies reveal that *pth2rb* is detectable in the developing zebrafish forebrain-midbrain in close proximity to PTH2. Functional investigation of *Pth2rb* stimulation by PTH2 shows a greater maximum response in intracellular cAMP compared to that of zebrafish *Pth2ra*. Together these studies provide the initial groundwork for further evaluations of the PTH2/PTH2R signaling interactions that may be applicable across species.

KEYWORDS: Development, parathyroid hormone, endocrine, neuroendocrine, G-protein coupled receptor

CHARACTERIZATION OF PTH TYPE-2 RECEPTOR (PTH2RB) AND ITS ENDOGENOUS
LIGAND PTH2 IN ZEBRAFISH DEVELOPMENT

NICHOLAS SEITZ

A Dissertation Submitted in Partial
Fulfillment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

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2019

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LIGAND PTH2 IN ZEBRAFISH DEVELOPMENT

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N.S.

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CHAPTER I: PARATHYROID HORMONE TYPE-2 RECEPTOR B

Introduction

The Parathyroid hormone (PTH) system has evolved in chordates to perform diverse functions including calcium homeostasis and signaling, embryonic development, reproductive physiology/behavior, nociception, and fear/anxiety responses (Cheloha et al. 2016; Suarez-Bregua et al. 2017). PTH, Parathyroid hormone 2 (PTH2, alias TIP39) and Parathyroid-like hormone (PTHLH, alias parathyroid hormone-related protein) interact with a family of class B G-protein coupled receptors (GPCRs) comprised of PTH1R, PTH1Rb/3R, and PTH2R (Rubin & Jüppner, 1999; see Table 1 for nomenclature key). PTH and PTHLH are able to bind and activate PTH1R to produce a cAMP response via activation of G_{α_s} (Gardella & Jüppner, 2001; Cheloha et al. 2016; Ehrenmann et al. 2018). PTH2R is able to bind PTH and PTH2 but displays different activation profiles between these two ligands (Usdin et al. 1999; Bisello et al. 2004). PTH2 stimulation triggers recruitment of PKC β and β -arrestin, receptor desensitization, and receptor internalization whereas PTH does not (Bisello et al 2004). The elevated potency displayed by PTH2 stimulation supports its role as PTH2R's primary ligand. PTH and PTHLH activity are involved in regulating renal fibrosis, chondrogenesis, nociception, bone development and maintenance in mammals and likely fish (Guo et al. 2017; Yan et al. 2012; Miao et al. 2012; Miao et al. 2008; Guerreiro et al. 2007; Shepherd et al. 2018; Gardinier et al. 2019; Hong et al. 2019; He et al. 2018; Nishimori et al. 2019; White et al. 2019). PTH is an important component in osteoporosis and kidney disease therapy (Swarthout et al. 2002; Chen et al. 2018; Mantovani and Elli, 2019). Deregulation of PTHLH contributes to humoral hypercalcemia of malignancy, intrahepatic cholangiocarcinoma, breast cancer, and disrupted T-cell development (Guerreiro et al. 2007; Mangin et al. 1988; Tran et al. 2018; Jia et al. 2017; Tang et al. 2017; Pinheiro et al.

2012). Neuroendocrine studies suggest PTH2R activity contributes to thermal sensitivity, spine disease, nociception, the fear/anxiety response, and inflammation (Dimitrov et al. 2011; Åkesson et al. 2015; Gellén et al. 2017; Dimitrov et al. 2013; Dobolyi et al. 2012; Tsuda et al. 2015). PTH2 has also been shown to influence bone development, keratinocyte differentiation and wound repair in the skin (Panda et al. 2012; Wilgus, 2017; Sato et al. 2016; Sato et al. 2017).

We have previously observed *pth2* and *pth2r* by *in situ* hybridization in early zebrafish development (Papasani et al. 2004; Bhattacharya et al. 2011). *Pth2* mRNAs can be detected at the beginning of the blastula stages (4 hpf), the brain during segmentation (22 hpf), and paired forebrain-midbrain domains by the hatching period (48-72 hpf). Detectable levels of *pth2r* can be seen very early during cleavage (1.75 hpf), throughout the CNS at 12 to 22 hpf, and becomes restricted to the midbrain-forebrain region and otic vesicles by 72 hpf. Here we analyze the fourth zebrafish PTH receptor homolog which we now refer to as *pth2rb* to distinguish it from the previously established zebrafish *pth2r* gene we will now call *pth2ra* (Papasani et al. 2004; Bhattacharya et al. 2011; Table 1). We report *pth2rb* isolation, syntenic and phylogenetic analysis, mRNA expression, and functional evaluation. We use the new *pth2rb* sequence and the recent crystal structure of PTH1R (Ehrenmann et al. 2018) to identify conserved specializations in the PTH2R family. Our aim is to provide initial characterization of an additional PTH2-sensitive receptor in zebrafish which will further the understanding of PTH2/PTH2R signaling in vertebrates.

PTH

Secretion and synthesis of PTH is coordinated by primarily by the parathyroid gland in humans, while supplementary secretion by the thymus has been observed in other mammals. The physiological role of PTH in mammals is maintenance of extracellular calcium homeostasis

and is utilized as a target in treatment of osteoporosis, chronic kidney disease, and maintenance of mesenchymal stem cell density (Risom et al. 2013; Frazier-Bowers et al. 2013; Ben-awadh et al. 2014; Chen et al. 2018; Tang et al. 2019). In zebrafish, which lack a parathyroid, PTH is synthesized at the brain, lateral line, and gills. The mature PTH hormone is an 84-residue polypeptide that is derived from pre-pro-PTH which includes a 25-residue extension at its N-terminus. Structurally, the first 34 residues of PTH (PTH (1-34)) are critical for binding and activation of target receptors (Castro et al. 2002; Ben-awadh et al. 2014, Wang et al. 2014). PTH acts in the bone, skin, and kidney via its PTH type-1 receptor (PTH1R) interactions to regulate serum calcium levels. When serum calcium levels are high, Ca^{2+} ions bind to calcium sensing receptors (CaSR) on the surface of the parathyroid to inhibit PTH secretion (Brown et al. 1993). At low calcium serum levels, PTH is secreted by the parathyroid to act directly at the bone and kidney, skin, and indirectly on the intestines. In bone, PTH binds to its target receptor, PTH1R, to initiate the activation of adenylate kinase leading to cAMP accumulation and subsequent phosphokinase A (PKA) pathway. In response to PKA pathway activity, cytokines are released by osteoblasts stimulating calcium, phosphate, and magnesium resorption from bone (Schluter, 1999; Guerreiro et al. 2007).

In the kidney, binding of PTH with its target receptors stimulates the phosphokinase C (PKC) pathway and activations of the 1α hydroxylase renal enzyme. Renal 1α hydroxylase then converts vitamin D to its active form to promote calcium uptake in the intestines (Garabedian et al. 1972; Fraser and Kodicek, 1973). More recently, examination of PTH activity in cutaneous tissue has shown that extracellular Ca^{2+} binds PTH1R and assists in sustaining the endosomal cAMP cascade (White et al. 2019).

PTHLH

The PTH-like hormone (PTHLH) was first isolated from neck, head, and lung tumors of patients suffering from humoral hypercalcemia of malignancy (HHM), a condition arising from adult T-cell leukemia/lymphoma (ATL) (Jans et al. 2003; Guerreiro et al. 2007). PTHLH possess a highly similar N-terminal domain with the first 34 amino acids of PTH and is able to bind equivalently to PTH receptors, PTH1R and PTH1Rb/3R, as PTH. PTHLH is required for development of teeth, bone, pancreas, and is highly expressed in cartilage during chondrogenesis (Yan et al. 2012; Nishimori et al. 2019). Loss of PTHLH function is embryonic lethal and yields abnormal musculoskeletal phenotypes, shortened epiphyseal cartilages, and increased ossification during bone remodeling (Lam et al. 2000; Strewler and Nissenson, 2003).

PTH1R and PTH1Rb/3R

Parathyroid hormone receptors (PTHRs) are a group of class B G-couple protein receptors (GPCRs) that exhibit a seven-(pass)-transmembrane domain. GPCRs are most commonly associated with sensory functions and mediating signaling by ligand interaction. Class B GPCR binding and activation are functionally separate events which rely on interactions between different domains of the ligand and its target receptor. GPCR binding affinity is mediated by interaction between the C-terminal domain of the ligand with the extracellular N-terminal domain of the receptor. Activation is reliant on the interface of the N-terminal domain of the ligand with the receptor's transmembrane helices (Bergwitz et al. 1996). As the largest class of membrane receptors in the human genome, they are the target for many drugs intended for clinical use (Overington et al. 2006).

Several rounds of genome duplication events are responsible for the diversity of members in the parathyroid hormone gene family across extant vertebrates today. Zebrafish possess a

number of duplicated paralogs that may be misidentified as ‘novel’ genes (Kang, 2004; Postlethwait, 2007). Zebrafish possess two genes that encode PTHRs, *pth1r* and *pth1rb/3r*, stimulated preferably by PTH or PTHLH. Mammals do not possess a *PTH1Rb/3R* homolog and the function of zebrafish *pth1rb/3r* remains unclear. The structural similarity of PTH1R and PTH3R indicate they are likely co-orthologs derived from gene duplication. Despite being co-orthologs the activation by PTH and PTHLH differs between PTH1R and PTH1Rb/3R. PTH1R is able to bind PTH, PTHLH, and PTH2 ligands, but only exhibits a cAMP response from PTH or PTHLH. PTH1Rb/3R binds PTH and PTHLH equivalently, but cAMP accumulation in response to PTH is significantly lower compared to PTHLH suggesting PTHLH as its primary ligand (Rubin and Jüppner, 1999).

PTH2

The peptide, parathyroid hormone 2 (PTH2) (also known as tuberoinfundibular peptide 39, TIP39) is a distant relative to PTH/PTHLH and was first isolated from bovine brain. Mature PTH2 is a 39 amino acid peptide and share similar sequence identity to PTH (Papasani et al. 2004). Study of CNS expression in rat tissues has shown PTH2 to be synthesized in the posterior thalamic areas, medial paralemniscal nucleus, and dorsal/dorsolateral hypothalamus (John et al. 2002; Dobolyi et al. 2003), while overall distribution is observed throughout the CNS (Dobolyi et al. 2003). In zebrafish, *PTH2* is detected at all stages, starting in early zygotes. Later, *PTH2* is restricted to the otic vesicles, heart, and the fore-midbrain boundary 48 hours post-fertilization (Papasani et al. 2004). PTH2 possesses activation and binding critical amino acid residues at positions 5 and 23, similar to PTH/PTHLH (Gardella et al. 1996). In COS-7 cells, human and mouse PTH2 has been shown to effectively bind and activate PTH2R to stimulate cAMP accumulation. Loss of PTH2 function by morpholino knockdown in zebrafish

results in lethality at 3 days post-fertilization. These morphants display small otic vesicles and eyes, hindbrain swelling, disrupted axon terminal patterning, and overall shorter body length in comparison to wild type zebrafish at similar stage in development (Bhattacharya et al. unpublished).

PTH2R

PTH2R was first identified in humans through its sequence similarity to PTH1R and ability to be stimulated by PTH (Usdin et al. 1995). However, the capacity for PTH to activate PTH2R is not conserved across species (Hoare and Usdin, 1999; Usdin et al. 1999; Dobolyi et al. 2003). The endogenous PTH2R ligand, PTH2 (TIP39), produces a robust cAMP response in human and rodent PTH2R-expressing cell lines (Usdin et al. 1999). Activation selectivity against PTH and PTHLH is mediated by interactions between Tyr-318 of PTH2R with the 5th residues of these two ligands (Weaver et al. 2017). Mammalian *PTH2* expression is detected at the subparafascicular area and medial paralemniscal nucleus in the adult brain (Dobolyi et al. 2003; Bago et al. 2009).

Results

Synteny and phylogenetic analysis of *pth2rb*

Several rounds of genome duplication events are responsible for the diversity of members in the parathyroid hormone gene family across extant vertebrates today. Zebrafish possess several duplicated paralogs that may be misidentified as ‘novel’ genes (Kang et al. 2004; Postlethwait, 2007). To identify the presence of a duplicate of zebrafish *pth2r*, we searched the zebrafish genomic database for *pth2ra*-like sequences and identified a putative coding region, *si:dkeyp-4h4.1* (XP_017208978), that shared 74% identity with zebrafish Pth2ra and 61% identity with human PTH2R in BLAST-aligned regions.

Isolation of the *pth2rb* ORF was performed by reverse transcription on whole zebrafish RNA with reverse primers complementary to the predicted *pth2rb* 3' UTR. Initial reverse transcription experiments produced a non-translatable *pth2rb* cDNA, with several premature termination codons in the expected signal peptide-coding region of exon 2. 5' RACE from exon 2 revealed a novel in-frame signal peptide-coding exon, now exon 1 of *si: dkeyp-4h4.1*. Full-length amplification of the intact *pth2rb* ORF was performed through PCR with forward and reverse primers complementary to the 5' and 3' UTRs. The amino acid sequence is shown aligned with all other human and *Danio* PTH receptors (Figure 1). Note that the receptor previously called PTH3R is encoded by ZFIN gene *pth1rb* and will thus be simply called PTH1Rb here, a name which better reflects the phylogeny (Table 1).

BLAST searches of the ENSEMBL zebrafish genomic database identify 13 exons of *pth2rb* on the reverse strand of chromosome 22 spanning contigs BX664720.7 and AL645755.2. By comparison, human *PTH2R* (NM_005048) also has 13 exons comparable to zebrafish *pth2rb*. Our previous work investigating the syntenic relationship between human and zebrafish co-orthologs showed that zebrafish *pth2ra* on Dre9 has neighboring sequences shared with human *PTH2R* on Hsa2 (Catchen et al. 2009; Bhattacharya et al. 2011). A similar comparison with the ENSEMBL database against zebrafish *pth2rb* on Dre22 indicates syntenic regions on Hsa2. The *pth2ra* and *pth2rb* genes reside in paralogous chromosome segments between chromosomes Dre9 and Dre22 respectively (Figure 2). Phylogenetic analysis indicates that Pth2rb groups basal to other fish PTH2Rs and could be inferred to function similar to other PTH2Rs (Figure 3). We conclude that *pth2ra* and *pth2rb* have the properties of conserved synteny with the human genome and paralogy within the zebrafish genome as expected if the two genes arose in the teleost genome duplication event.

Evolutionary structural analysis indicates ligand binding site remodeling

With two divergent forms of each receptor present in fish, we were able to investigate how the PTH1R and PTH2R groups have specialized at the structural level. All six PTH1R and PTH2R proteins from human and *Danio* are aligned in Figure 1. Overall conservation is high, especially in the 7-TM helix bundle and the ligand-binding ECD. When we shade the conserved residues at a 50% level, 30 "split consensus" positions become apparent (green) at which PTH1R proteins and PTH2R proteins are each conserved within their group, but the groups have adopted different identities or different amino acid types. These split consensus cases offer a proxy for positions that are structurally/functionally important, but where a structural specialization has occurred between PTH1R and PTH2R. We sought to correlate these 30 positions with other features of the receptor. The alignment notes 21 ligand binding residues of PTH1R (asterisks), as defined by Ehrenmann et al. (2018). Most (13 of 21) of these ligand binding residues are perfectly conserved in the alignment, but 4 are split consensus residues, and another 8 are directly adjacent to split consensus residues. Most of the ligand binding residues fall into five concentrated clusters along the sequence, and all of those sequence segments include split consensus residues. The same is not true for the conserved intra-membrane polar network amino acids (the six residues in red in Figure 1; Ehrenmann et al. 2018), none of which are within 3 a.a. of a split consensus residue. The split consensus residues were mapped onto the PTH1R crystal structure in green in Figs. 4-5 (Ehrenmann et al. 2018). 90% of the split consensus positions occur in the 7-helix bundle and loops, and 50% occur along the segment TM6-ECL3-TM7, which forms one flank of the PTH binding pocket (arrow, Figure 4). ECL3 also has a PTH2R-specific deletion of one aa. Figure 5 shows the orientations of those specialized side chains; most lie along the binding pocket. One notable switch is that PTH1R Y429, a key ligand binding

residue in position to H-bond with PTH, is converted to a conserved His in PTH2R proteins. Taken together, the results support a model in which the overall structure and signal transduction mechanism were maintained in vertebrate evolution while the lower PTH binding pocket was remodeled to provide for different ligand binding profiles in PTH1R vs PTH2R (see below).

Developmental expression pattern has diverged in the *pth2r* paralogs

To evaluate the expression of *pth2rb* we investigated the spatio-temporal distribution of *pth2rb* by whole mount *in situ* hybridization throughout development. Similar stages of development were also observed for expression of *pth2ra*, *pth2*, and *pth* for comparison (Figure 6-8). The earliest *pth2rb* transcripts were observed accumulating in the blastula at 4 hrs post-fertilization (hpf) (Figure 6B). At 12h, expression of *pth2rb* was detected during early segmentation throughout the developing central nervous system (CNS) (Figure 6C). During segmentation (17h-24h) *pth2rb* begins to primarily be expressed in the brain (Figure 6D, 6E). Through the hatching period (48h-72h), *pth2rb* becomes restricted to paired domains at the forebrain-midbrain regions (Figure 6G, 6H). Coronal sections of the midbrain-forebrain boundary show *pth2rb* expression in paired domains in the ventral posterior tuberculum (PTv) (Figure 7I) at 2dpf. At 3dpf, *pth2rb* is observed across broader paired domains at the ventral posterior tuberculum (PTv) and the dorsal posterior tuberculum (PTd) (Figure 7J).

We found the expression patterns of the two paralogs *pth2ra* and *pth2rb* diverge sharply in later development. Both are expressed broadly throughout the CNS at early developmental stages, but *pth2ra* becomes restricted to the midbrain-hindbrain and otic vesicles at later stages (Figures 8C-8F), while *pth2rb* is expressed robustly at the forebrain-midbrain region (Figures 6G, 6H, 7I, 7J), in closer vicinity to *pth2* and *pth1b* mRNAs (Figure 7C-7F) (Bhattacharya et al.

2011). By 72 hpf, the *pth2ra* and *pth2rb* probes show distinct and non-overlapping patterns of high-expressing cells in the brain (Figures 6G, 6H vs. 8C-F).

Pth2rb is a functional PTH2 receptor

As members of the class B GPCR family, PTH receptors signal via the $G\alpha_s$ -cAMP pathway. To analyze PTH2Rb stimulation in response to ligands PTH (1-34), PTHLH (1-36), and PTH2 relative to other parathyroid receptors, cAMP accumulation was measured via the GloSensor lucifase-based reporter assay. Transfected HEK293-derived (GS-22A) cells stably expressing zebrafish Pth2rb, zebrafish Pth2ra, human PTH1R, or human PTH2R were incubated with varying concentrations of ligand. Maximum luminescence (counts per second or cps $\times 10^{-3}$) from receptor-dependent cAMP activity was measured following increasing concentrations of ligand exposure ([Ligand], Log (M)). Human PTH1R was observed to be stimulated by PTH (1-34) and PTHLH (1-36) (Figure 9A). PTH2 failed to produce strong cAMP accumulation with human PTH1R (Figure 9A). Human PTH2R showed a robust cAMP response to PTH2 exposure and near equivalent E_{Max} when exposed to higher concentrations of PTH (1-34) (Figure 9B; Table 2). Zebrafish Pth2ra exhibited robust cAMP-dependent luminescence in response to PTH2 exposure and was stimulated by higher concentrations of PTH (1-34) and PTHLH (1-36) to relatively half the E_{Max} observed from PTH2 (Figure 9C). Stimulation of Pth2rb by PTH2 displays approximately 10-fold and 2-fold E_{Max} compared to zebrafish Pth2ra and human PTH2R respectively (Figure 9D). Pth2rb also was able to generate a lower cAMP response from PTH exposure, reaching an E_{Max} to approximately half of that observed in human PTH1R and PTH2R. Thus, PTH2 is likely the primary ligand of zebrafish Pth2rb but does appear to be able to be partially stimulated by PTH (1-34) similar to Pth2ra.

Discussion

In this study we have reported the cDNA cloning and characterization of a previously undescribed PTH2-sensitive class B GPCR in zebrafish, encoded by *si: dkeyp-4h4.1*. This is the 4th and final member of the PTH receptor family in zebrafish, necessitating a change in gene names for this family. The *pth2r* gene should be designated *pth2ra*, and the paralog described here should be renamed from *si: dkeyp-4h4.1* to *pth2rb*. The PTH receptor family in zebrafish is thus Pth1ra, Pth1rb (previously 3r), Pth2ra, and Pth2rb (Table 1). We note that the 1ra/b and 2ra/b designations do not reflect parallel evolutionary histories; the 1ra/b pair arose in an early event, perhaps the 2nd vertebrate whole genome duplication (Pinheiro et al. 2012), while the 2ra/b pair apparently arose in the 3rd, teleost-specific whole genome duplication. Zebrafish *pth2rb* locus exhibits syntenically conserved sequences with the human *PTH2R* ortholog as well as with the zebrafish *pth2ra* paralog. This syntenic conservation indicates that *pth2rb* is likely an appropriate ortholog for inference of potential mammalian *PTH2R* function.

Compared to Pth2ra, Pth2rb possesses a 56 a.a. insertion in the C-terminal intracellular domain (Figure 1). The presence of a relative insertion here is not *Danio*-specific; it is found across numerous species of ray-finned fishes, but its sequence conservation is weak. This extended C-terminus may allow for recruitment of additional GPCR accessory proteins to form complexes and further optimize or tune signaling, possibly through endosomal incorporation similar to that observed in PTH1R (Vilardaga et al. 2012; Bisello et al. 2004; Ferrandon et al. 2009; Culhane et al. 2015; Bockaert et al. 2003; Hall and Lefkowitz, 2002; Halls, 2018; Thompson et al. 2014).

In the sequence analysis (Figure 1) we sought use PTH1R/2R evolution to highlight, in an unbiased manner, potential sites of functional specialization. The ligand binding pocket,

particularly at TM6-7, stands out as major site of remodeling early in chordate-vertebrate evolution (predating fish-mammal divergence) (Figures 4-5). Indeed, our analysis recovered three residues that were previously established by mutagenesis studies to provide discrimination between ligands. The TM7 segment C₃₉₇ELF₄₀₀ (hPTH2R numbering) has three split consensus residues; two of these (C₃₉₇ and F₄₀₀) are responsible for PTH2R's ability to discriminate between the PTH and PTHLH ligands, as switching either one to the PTH1R form allows PTHLH to activate the receptor (Turner et al. 1998). The split consensus residue in TM3, L289 in PTH1R /I244 in PTH2R, also has this property (Bergwitz et al. 1997; Turner et al. 1998). As seen in Figure 5, L289 faces the ligand binding pocket, positioned such that the small steric change from L to I can alter the pocket. In similar mutagenesis experiments, Weaver et al. (2017) showed that PTH2R specificity for PTH2/TIP39 depends largely on its Y318 residue (matching PTH1R I363; arrowhead in Figure 1). Y318 did not meet our criteria for a split consensus residue because, interestingly, zebrafish Pth1rb shares the Tyr residue at this position with PTH2R, making its ligand binding pocket more "PTH2R-like". Zebrafish Pth1rb appears to have evolved to mimic Weaver's in vitro mutagenesis result, since Pth1rb can be activated by PTH2 ~10-fold better than Pth1ra (Gensure et al. 2004).

Our analysis of *pth2rb* expression by *in situ* hybridization indicates that mRNAs are expressed very early in zebrafish embryos through 72 hrs past fertilization. Zebrafish *pth2rb* expression is observed beginning at 4 hpf throughout the early developing CNS and exhibits partial overlap with that observed for *pth2ra*, however mRNA levels appear to vary by 24hr between these paralogs. At 48 hpf we observed that *pth2rb* mRNAs are restricted to the forebrain-midbrain region of the brain and no longer overlap with *pth2ra* expression by 72 hpf.

Pth2rb expression at 72 hpf also appears to be present across a wider range of tissue at the forebrain versus the otic vesicles and adjacent nuclei expression of *pth2ra* in the hindbrain.

Further analysis of *in situ* coronal sections reveals that *pth2rb* mRNAs are expressed 3 dpf primarily in tissues at the developing dorsal thalamus (DT) and posterior tuberculum (PT). We also observe *pth2* ligand mRNA expression in cells adjacent to *pth2rb* mRNAs at the PT at 2 dpf and 3dpf. In zebrafish, the forebrain region is involved with stress experience, memory, and social behavior (Stednitz et al. 2018; Huang et al. 2019). The zebrafish PT shares characteristics with the dopaminergic A9 and A12 neurons in the mammalian brain (Blin et al. 2008; Matsui and Sugie, 2017, Mueller et al. 2011). While mammals lack a direct counterpart to the posterior thalamus, prosomeres two and three of the mammalian forebrain give rise to homologous regions in development (Mueller and Wullimann, 2003; Mueller, 2012; Wullimann and Puelles, 1999). Zebrafish lack a complex isocortex but their dorsal thalamic region does spatially correspond to the isocortex in mammalian brains (Mueller, 2012; Lodato and Arlotta, 2015). The prevalence of *pth2rb* at the forebrain suggests that it may play a regulatory with pituitary associated genes such as *pitx3*, *gnrh2*, and *shh* during early development (Herzog et al. 2003). Additional functional investigation of *pth2rb* in zebrafish neural tissues or behavioral studies may provide further insight to regulation and development of mammalian central nervous system.

Functional analysis of cAMP stimulation in response to PTH ligands shows that Pth2rb differs from Pth2ra not just in expression pattern, but also in ligand bias. Like human PTH2R, zebrafish Pth2rb shows greater activation by PTH than PTHLH, while Pth2ra is activated by both PTH and PTHLH to similar extents. Pth2ra, Pth2rb, and human PTH2R all produce the most robust cAMP accumulation after treatment with PTH2, while human PTH1R is not activated by PTH2. Presentation of PTH2 to Pth2rb yields a maximal cAMP response over 10-

fold greater than that observed in zebrafish Pth2ra and approximately 2-fold greater than human PTH2R. The large difference in cAMP response observed between Pth2ra and Pth2rb by PTH2 would indicate significant differences in binding efficacy, accessory protein interactions, G-protein regulators, and other factors that mediate GPCR signaling. One mechanism that may be of particular interest is the ability of GPCRs to mediate G-proteins from endosomal complexes with their ligands. The endosomal internalization of these ligand-receptor complexes along with several accessory proteins have been observed previously in PTH1R signaling to promote sustained cAMP production (Daaka et al. 1998; Ferrandon et al. 2009; Vilardaga et al. 2009; Eichel and von Zastrow, 2018; Calebiro et al. 2009; Vilardaga et al. 2014; Tsvetanova et al. 2015). Endosomal incorporation in response to PTH2 exposure been previously reported in human PTH2R, but the full functional significance for endocytosis has remained unclear (Bisello et al. 2004). In some cases, endosomal GPCR activation allows for signaling to persist beyond initial ligand binding and may be used as a means of resensitization if the receptor can be reincorporated at the membrane (Tsvetanova et al. 2015). The extended C-terminal domain of zebrafish Pth2rb may be a point of interest to identify factors associated with GPCR-endosomal signaling such as GPCR-associated sorting protein (GASP) and endosome sorting complex required for transport (ESCRT) multi-protein (Whistler et al. 2002; Katzmann et al. 2001; Tsvetanova et al. 2015).

These results provide an initial characterization of the PTH2-sensitive receptor, Pth2rb, isolated in zebrafish and member of the parathyroid family of class B GPCRs. Identified through a genomic homology screen against zebrafish *pth2ra*, Pth2rb displays an extended C-terminal domain. Observations from *in situ* hybridizations show that *pth2rb* likely has a role in early forebrain-midbrain development where it is expressed adjacent to tissues expressing *PTH2* and

pth1b ligands. Pth2rb is able to produce a notably higher maximum cAMP response than observed in Pth2ra when exposed to PTH2. Future study into the function and expression of Pth2rb will broaden our understanding of vertebrate PTH2 signaling.

Figure 1. Alignment of *Danio* and Human PTHR family members. Major structural elements (Pioszak & Xu 2008; Ehrenmann et al. 2018) are indicated: The extracellular domain's alpha helix 1 (helix icon) and beta sheet fold (gray bar); the seven transmembrane (TM) helices (TM1-7, yellow bars); intracellular loops ICL1-3; and four pairs of disulfide bonded extracellular Cys residues (individual disulfides indicated by color-matched pairs of stars). Main residues of the TM bundle's intramembrane central polar network are highlighted red (Ehrenmann et al. 2018).

Identities (black) and similarities (gray) are highlighted at a 50% conservation level. At 30 positions the amino acid type is conserved within PTH1R and within PTH2R, but differs between them, indicating a conserved specialization of PTH1R vs. PTH2R. These "split consensus" residues are noted in dark green (if PTH1R and PTH2R have different identities) or light green (if PTH1R and PTH2R use different similarity groups). Only positions R162, V197, and A369 have two different residues at 50% identity, but mixed across the PTH1R and PTH2R groups (indicated by white bars). The ligand binding residues discussed by Ehrenmann et al. (2018) are marked by an asterisk. Note that 19 of these 21 ligand binding residues lie near (within 4 a.a. of) a split consensus residue, indicating the ligand binding pocket has been customized in PTH1R vs. PTH2R. Arrowhead notes the ligand-discrimination residue Y318 in PTH2R (Weaver et al. 2017).

Figure 2. Syntenic analysis of *pth2rb* orthologs and paralogs. Comparison of neighboring genes between zebrafish *pth2rb* on Dre22 with human *PTH2R* on Hsa2 (A) or zebrafish *pth2ra* on Dre9 (B). Green boxes indicate conserved orthologs or paralogs genes. White boxes indicate non-conserved peripheral genes. Colored connecting lines represent subclusters of neighboring genes.

Figure 3. Phylogenetic analysis of PTH receptor family. Phylogenetic relatedness between PTH receptor orthologs across vertebrates with vasoactive intestinal peptide (VIP) genes as the outgroup. Top grouping with other *PTH2R* orthologs suggests *pth2rb* arose from a duplication event some point after the tetrapod-fish divergence.

Figure 4. Crystal structure of PTH1R (PDB 6FJ3; Ehrenmann et al. 2018). The two views differ by a $\sim 90^\circ$ rotation around the long axis. The modified PTH hormone used in the crystal is shown surface-rendered in purple. Dotted lines indicate approximate position of the plasma membrane. The "split consensus" residues that are highlighted in green in Fig. 1 indicate points of intra-family conservation but inter-family divergence/specialization between PTH1R and PTH2R. Those residues are shown here in green sphere-rendering on the PTH1R backbone trace; select residues are labeled (PTH1R numbering). Note that this PTH1R/PTH2R specialization is particularly concentrated on one side of the intramembrane PTH binding pocket (arrow).

Figure 5. Close-up of PTH1R crystal structure (PDB 6FJ3; Ehrenmann et al. 2018). The region indicated by the arrow in Fig. 4 is shown, with the split-consensus residues in green cylinder-rendering and PTH with purple sphere-rendering. Most of the diverged side chains are in a position to potentially alter the PTH pocket surface. Ligand-discrimination residue L289 (I244 in PTH2R) is noted (Bergwitz et al. 1997; Turner et al. 1998).

Figure 6. *In situ* hybridization detection of *pth2rb*. Detection of tissue-specific expression of *pth2rb* mRNA transcripts in whole mount *in situ* hybridization by cRNA probe. *Pth2rb* expression is observed in early cleavage through hatching period (A-H) in the CNS, forebrain (f), midbrain (m), hindbrain (h), retina (r), and notochord (no). Magnification of panel G and H are 2X of others.

Figure 7. Coronal cryosections *in situ* hybridizations to detect *pth* ligands and receptors.

pth1a is not detected in the brain at 2 dpf or 3 dpf (A, B). *pth1b* is observed in paired domains in the dorsal posterior tuberculum (PTd) (C, D). *pth2* is observed at 2 dpf and 3 dpf in the (PTd) (E, F) similar to *pth1b*. *pth2ra* is expressed 2 dpf at the otic vesicles (ov) (G) and adjacent midbrain-hindbrain tissues at 3 dpf (H). *pth2rb* is detectable at 2 dpf at the ventral posterior tuberculum (PTv) (I) and (PTd) at 3 dpf (J). DT: Dorsal thalamus, Po: Preoptic region

Figure 8. Whole mount *in situ* hybridization detection of *pth2ra*. Detection of zebrafish *pth2ra* mRNA transcripts by whole mount *in situ* hybridization. *Pth2ra* is strongly expressed throughout the brain at the pharyngeal period (A). *Pth2ra* expression becomes restricted to paired domains beginning at the hatching period in the otic vesicles (ov) and mid-hindbrain region (B-F). f: forebrain, m: midbrain, h: hindbrain.

Figure 9. Evaluation of cAMP activity by PTH ligands. cAMP ligand-dose-response analysis of PTH (1-34), PTH2, or PTHLH (1-36) by HEK-293 cells stably transfected with luciferase based GloSensor reporter. cAMP signaling assessed in cells expressing either (A) human PTH1R (hPTH1R), (B) human PTH2R (hPTH2R), (C) zebrafish Pth2ra (zPTH2Ra), or zebrafish Pth2rb (zPTH2Rb). Data are expressed as means ($\pm$ SEM). Curves were fit to the data using a four-parameter, non-linear regression function.

Table 1**Nomenclature of PTH ligands and receptors.**

Type	Earlier or preliminary name	Current or proposed name
<i>Danio</i> gene	<i>zPTH1R</i>	<i>pth1ra</i>
<i>Danio</i> gene	<i>zPTH3R</i>	<i>pth1rb</i>
<i>Danio</i> gene	<i>pth2r</i>	<i>pth2ra</i> (proposed)
<i>Danio</i> gene	<i>si: dkeyp-4h4.1</i>	<i>pth2rb</i>
PTH family hormone	TIP39	PTH2
PTH family hormone	PTHrP	PTHLH

Table 2**Measurements of GloSensor cAMP-dependent luminescence in transfected HEK-293 cells.**

Receptor	Ligand	pEC ₅₀	E _{MAX} (CPS X 10 ⁻³)
hPTH1R	PTH (1-34)	9.48 ± 0.13 0.33 nM	63 ± 17
	PTH LH (1-36)	9.58 ± 0.13 0.26 nM	64 ± 18
	PTH2	6.35 ± 0.26 446 nM	5.1 ± 1.0
hPTH2R	PTH (1-34)	9.07 ± 0.13 0.85 nM	66 ± 18
	PTH LH (1-36)	6.26 ± 0.27 546.26 nM	61 ± 25
	PTH2	9.69 ± 0.10 0.21 nM	66 ± 17
zPth2ra	PTH (1-34)	7.97 ± 0.23 10.73 nM	4.2 ± 1.3
	PTH LH (1-36)	8.17 ± 0.14 6.83 nM	4.5 ± 1.2
	PTH2	8.35 ± 0.11 4.46 nM	8.3 ± 1.3
zPth2rb	PTH (1-34)	7.63 ± 0.05 23.69 nM	33 ± 4
	PTH LH (1-36)	n.d. n.d.	n.d. n.d.
	PTH2	8.96 ± 0.13 1.11nM	116 ± 25

Table 3**P-values of zPth2rb vs. zPth2ra**

Ligand	pEC ₅₀	E _{MAX} (CPS X 10 ⁻³)
PTH (1-34)	0.21	0.002
PTH LH (1-36)	N.D.	N.D.
PTH2	0.008	0.013

CHAPTER II: PEZ

Introduction

Protein tyrosine phosphatases (PTPs) are responsible for the removal of phosphates from tyrosine residues in proteins. The enzymatic tyrosine de-phosphorylation of PTPs acts opposite from protein tyrosine kinases (PTKs) that post-transcriptionally modify target proteins by attaching phosphates to tyrosine. The combination of phosphorylation and de-phosphorylation regulate signaling molecules, many of which are involved in cell cycle regulation, growth, differentiation, and protein turnover.

The PTP, Pez (PTPN14), was first isolated from human breast tissue in a screen for novel PTPs. The human form of Pez (PTPN14) is an 1187 residue protein with a molecular mass of 130 kDA and can be broken into three functional N-terminus, central linker, and C-terminus (Smith et al. 1995). *Drosophila* Pez and its mammalian homologs PTPHD1/PTPN21 and PTPD2/PTP36/PTPN14 share similar structure in regard to their N-terminal FERM and C-terminal PTP domains, while their central linker regions show high amounts of variation (Smith et al. 1995; Edwards et al. 2001; Poernbacher et al. 2012). The N-terminal domain of Pez contains a 4.1, ezrin-radixin, moesin (FERM) domain that allows interface with the plasma membrane of the cell and elements of the cytoskeleton. As a regulatory element of the actin cytoskeleton, Pez forms complexes with focal adhesion kinase (FAK) and Src tyrosine kinase to promote cell motility in response to growth factors (Carlucci et al. 2008). At the cell membrane Pez is able to dephosphorylate Beta-catenin to stabilize cell-cell adhesions and form transient signaling complexes with epidermal growth factor receptor (EGFR) (Wadham et al. 2003; Carlucci et al. 2010; Roda-Navarro and Bastiaens, 2014). Pez overexpression has been associated with perturbations of the actin cytoskeleton, abated cell growth, and reduced focal

adhesions (Ogata et al. 1999; Wadham et al. 2003; Wyatt et al. 2007). The lack of a signal sequence or transmembrane domain indicates that Pez does not directly embed into the membrane and remains cytoplasmic (Smith et al. 1995). The central linker domain contains proline-rich motifs resembling an SH3 domain and a WW domain that may also assist in Pez conformation in vivo (Smith et al. 1995). The presence of an N-terminal FERM domain and multiple protein interface motifs at the central linker domain suggest that Pez may act as a scaffold that promotes interactions between multiple other proteins.

The PTP domain of Pez would suggest that overexpression would lead to increased phosphatase activity on targets with phosphorylated tyrosine (pTyr). This predicted outcome is observed in Pez's interaction with Beta-catenin to stabilize cell-cell adhesions (Wadham et al. 2003). However, follicular cells of *Drosophila* embryos overexpressing Pez actually exhibit an increase of pTyr proteins. In affected follicle cells, Pez and pTyr aggregates can be detected at the apical membrane in close proximity by immunofluorescent antibodies. While Pez aggregates are observed at the apical membrane in follicle cells, they fail to bind several factors commonly associated there including: cadherin/catenin complex, actin, moesin, and spectrin. Ectopic Pez is shown to actually displace spectrin and F-actin at the apical membrane and coincides with adhesions forming between the oocyte and the apical membrane of follicle cells (Edwards, unpublished).

Absence of a kinase domain would suggest that at high levels Pez indirectly contribute to tyrosine phosphorylation likely by assisting activation of one or more kinases. The ability for Pez to act as a scaffold protein at the membrane may promote more kinase interactions with their targets at the apical membrane leading to the build-up of pTyr observed. If the activity of an individual PTK is responsible for the pTyr accumulation associated with ectopic Pez, we would

expect that the knockdown of said PTK would result in an observable decrease in pTyr accumulation at the apical membrane. To identify PTK activity associated with Pez, we performed a knockdown screen in which RNAi lines targeting individual candidate PTKs were crossed to a line able to ectopically express Pez. The initial candidate PTKs to be knocked down were *Abl*, *Ack*, *Csk*, *Shark*, *Src42A*, and *Src64B*. These kinases were chosen for their association with establishment of cell polarity, apical membrane, oogenesis, or the Hippo pathway (Ferrante et al. 1995; Tamada et al. 2012, Hu et al. 2016; Kwon et al. 2015; Thomas and Wieschaus, 2004; Zandy et al. 2007). Our initial most likely candidate was *Abl*, the leukemia oncogene, which is expressed early on in egg chamber development, associates with F-actin, is found at the apical membrane. *Abl*'s structure is often described as a 'clamp' that when inactive remains closed via an auto-inhibitory interaction between its N-terminal SH2/SH3 domains with its C-terminal lobe regions. Unclamping of *Abl* via myristolation of its N-terminus allows for conformational rearrangement which exposes its SH3 domain for potential interactions with the proline-rich central linker of Pez, which is a critical step for activating *Abl*'s SH2 domain's kinase activity (Hantschel, 2012). Crosses allowed for the evaluation of pTyr accumulation in egg chamber follicle cells simultaneously expressing ectopic Pez and RNAi against a PTK via a clonal FLP-out approach.

The possibility of two or more PTKs responsible for pTyr accumulation complicates this approach, as RNAi combinations to be expressed with ectopic Pez greatly increases the number of crosses performed and time to generate data. If no detectable inhibition of pTyr accumulation is observed in individual PTK RNAi cross this would indicate multiple PTKs are active at the apical membrane or an increase of PTK activity is not the mechanism behind the pTyr.

Results

We searched the genome to identify all tyrosine kinases that could be targets of Pez. A total of 32 tyrosine kinase genes (excluding dual-specificity kinases) were identified by an exhaustive BLAST analysis of *Drosophila* coding regions using several known tyrosine kinases as "probes". The list does not include dual-specificity kinases. Kinases tested in this screen in bold. (-) represent low or no ovary expression based on RNA-seq data (Brown et al. 2014) (Table 1). Kinases that are not expressed in ovaries (flybase.org) were excluded from further consideration.

To produce flies in which Pez is clonally overexpressed along with loss of PTK expression, we generated the stock [*Hsp70-FLP*], [*Act5C>CD2>GAL4*]; [*GMR-GAL4*], [*UAS-Pez*]; [*UAS-mCD8-mCherry*]. This line can be crossed to any UAS-RNAi stock, and the progeny assessed for differences in Pez or pTyr antibody staining. In this system, *Hsp70* controls expression of FLP recombinase which can be 'turned on' by heat shock treatment. When expressed, the FLP recombinase excises the FRT-flanked *CD2* spacer DNA upstream of *GAL4*, allowing for its expression via the cytoplasmic actin promoter. Accumulation of *GAL4* in these clonal cells leads to activation of UAS-controlled *CD8-mCherry* RFP marking the clones, RNAi hairpin of the kinase knockdown line crossed, and Pez overexpression. Ovaries with UAS-Pez and UAS-TRiP RNAi elements were dissected from females ~60hr post-heat shock to evaluate Pez and pTyr protein profiles. Pez and pTyr protein aggregates were observed using rabbit-anti-Pez and mouse-anti-pTyr antibodies detected by anti-rabbit-647 and anti-mouse-488 fluorescent antibodies respectively.

Confocal images were collected from stage 8-10 ovarioles from the UAS-Pez line crossed to kinase RNAi lines (Fig. 1). No visible decrease in pTyr co-localization with Pez was observed

in *Abl*-RNAi (Fig. 1A-F), *Ack*-RNAi (Fig. G-I), *Csk*-RNAi (Fig. 1J-L), *Shark*-RNAi (Fig. 1M-O), or *Src64B*-RNAi (Fig. 1P-R) lines. A decrease in pTyr was observed in RNAi knockdown of *Src42A* B-55868 (Fig. 2M-O & S.2), that targets exon 1 of the *Src42A-RA* isoform (FBtr0335276) which contains an SH3 domain unique to this transcript. Two additional *Src42A* RNAi lines targeting *Src42A-RA* (B-02755 & B-10817) (Fig. 2A-C & 2P-R), failed to produce a visible difference in pTyr accumulation. Specific RNAi targeting *Src42A-RB* (Fig. 2D-F) or the shared conserved C-terminal domain of *Src42A-RA/-RB* (Fig. 2G-I) also did not appear to inhibit pTyr accumulation. The pTyr of junctions between clonal follicle cells and neighboring wild-type cells do not appear to be affected, however the increase of pTyr in clonal cells may make differences present difficult to resolve.

Discussion

To investigate the pTyr accumulation in cells overexpressing the Hippo pathway member, *Pez*, we observed effects of kinase RNAi on *Pez* overexpressing follicle cells. Our crosses simultaneously overexpressing *Pez* and cytoplasmic tyrosine kinase knockdowns indicate that one *Src42A* RNAi line (B-55868) was sufficient to visibly reduce pTyr accumulation in clonal follicle cells. This RNAi line specifically targets the *Src42A-RA* isoform, the gene's major transcript in adult females (flybase.org). However, two other lines targeting this isoform failed to visibly inhibit pTyr accumulation; this may be due to low efficacy RNAi knockdown.

The inability of additional *Src42A* RNAi lines to inhibit pTyr accumulation suggests that the specific 5'UTR of *Src42A-RA* targeted by B-55868 is the most effective at knocking down expression of this kinase. While some RNAi lines may show low or variable levels of suppression, all crosses of *Src42A* RNAi lines tested in our screen with ectopic *Pez* exhibit strong wing phenotypes when crossed to *ms1096-GAL4* (not shown). The presence of these

wing phenotypes makes the possibility of these other *Src42A* RNAi lines being inactive highly unlikely. A BLAST query with the 21-nt RNAi target sequence of B-55868 shows no significant identity with other transcripts in the *Drosophila* genome. There are no other matches of 20 or 21 bases, and just two matches of 19 bases (to *couch potato* and *SK*), neither of which has significant ovary expression. Thus, the pTyr inhibition observed is likely not a result of off-target RNAi.

The hypomorphic nature of RNAi may provide difficult evaluation of the elevated pTyr levels exhibited by follicle cell expressing ectopic *Pez*. In the case of *Pez* overexpression, if a kinase's activity increased several fold compared to WT, incomplete knockout may be insufficient for visually detectable decrease in pTyr proteins. Therefore, while other RNAi lines could significantly decrease a given kinase's expression, the hyperactivity of any remaining kinase may make these RNAis appear ineffective when simply visualized. A quantitative approach measuring signal intensity, size, or abundance of pTyr aggregates may prove useful to determine knockdown effectiveness. However, these parameters vary with the stage of the egg chamber and age of the clone, making quantitation difficult in that tissue.

An alternative approach for kinase ablation could be through the popular CRISPR/Cas9 system to examine lines that can conditionally generate double stranded breaks (DSB) to target kinase genes. Flies that possess the *UAS-Cas9* element and capable of conditional *GAL4* expression can be crossed with flies expressing sgRNA for a specific kinase. Like our current kinase knockdown screen, *GAL4* expression would generate clonal follicle cells that induce a kinase knockout mediated by Cas9-sgRNA.

Future work in better understanding apical pTyr accumulation will include detection of *Src42A* in cells overexpressing *Pez*. The ability to detect *Src42A* will allow us to determine the

extent that Src42A can be knocked down by RNAi lines when compared to wild-type. Detection will also be helpful in determining if Src42A is being directly bound to Pez at the apical membrane or if it remains cytosolic. Unfortunately, a preexisting Src42A line with a detectable marker or epitope tag is not currently available and would have to be constructed. Three monoclonal antibodies derived from human Src42A (AFFN-SRC-1D2, 1E5, and 9C1) fail to show binding in a Src42A-specific pattern in *Drosophila* and would not be appropriate for this model.

Pez is a conserved PTP associated with regulation of the Hippo signaling pathway. Ectopic Pez expression in the *Drosophila* follicle cell model results in an unexpected increase of pTyr colocalized with Pez aggregates at the apical membrane. To determine if the activity of a PTK was responsible for pTyr accumulation we performed a screen in which we could observe follicle cells simultaneously expressing a PTK RNAi in the presence of ectopic Pez. Analysis of these PTK knockdown crosses indicates that RNAi targeting the 5'UTR of *Src42A-RA* isoform is sufficient to inhibit pTyr accumulation. This inhibition appears specific to this form as knockdown of *Src42A-RB* fails to produce a similar inhibition. This suggests that Src42A-PA is likely associating with Pez at the apical membrane where it can phosphorylate protein targets more frequently or is itself activated by Pez. Our observations on the pTyr relationship between Pez and knockdown of *Src42A* kinase indicates that *Src42A* may play an indirect role in the Hippo signaling pathway.

Figure 1. Accumulation of Pez and pTyr proteins in the presence of kinase RNAi. Pez overexpressing clonal follicle cells display Pez aggregates co-localized with pTyr proteins at the apical membrane. Secondary anti body detection of Pez and pTyr in UAS-Pez crossed to: Abl-RNAi BL35327 (A-C), Abl-RNAi BL61170 (D-F), Ack-RNAi (G-I), Csk-RNAi (J-L), Shark-RNAi (M-O), Src64B-RNAi (P-R). Overlay of Pez (red), pTyr (green), mCD8-RFP clone marker (blue).

Figure 2. Accumulation of Pez and pTyr proteins in the presence of Src42A RNAi. Pez overexpressing clonal follicle cells display Pez aggregates co-localized with pTyr proteins at the apical membrane. Secondary anti body detection of Pez and pTyr in UAS-Pez crossed to: Src42A-RNAi 02755 (A-C), Src42A-RNAi 8338 (D-F), Src42A-RNAi 26019 (G-I), Src42-Df 35990 (J-L), Src42A-RNAi 55868 (M-O), Src42A-RNAi 10817 (P-R). Overlay of Pez (red), pTyr (green), mCD8-RFP clone marker (blue).

Table 1**Complete, nonredundant set of standard tyrosine-specific kinases in *D. melanogaster*.**

Abbreviation	Full gene name	CG number	FlyBase record #
Abl	Abl tyrosine kinase	CG4032	FBgn0000017
Ack	Activated Cdc42 kinase	CG14992	FBgn0028484
Ack-like	Activated Cdc42 kinase-like	CG43741	FBgn0263998
Alk (-)	Anaplastic lymphoma kinase	CG8250	FBgn0040505
Btk29A	Btk family kinase at 29A	CG8049	FBgn0003502
Btl (-)	Breathless	CG32134	FBgn0285896
Cad96Ca (-)	Cadherin 96Ca	CG10244	FBgn0022800
CG3277 (-)	CG3277	CG3277	FBgn0031518
Csk	C-terminal Src kinase	CG42317	FBgn0262081
Ddr (-)	Discoidin domain receptor	CG33531	FBgn0053531
Dnt	Doughnut on 2	CG17559	FBgn0024245
Drl (-)	derailed	CG17348	FBgn0015380
Drl-2 (-)	Derailed 2	CG3915	FBgn0033791
Egfr	Epidermal growth factor receptor	CG10079	FBgn0003731
Eph	Eph receptor tyrosine kinase	CG1511	FBgn0025936
Fak	Focal adhesion kinase	CG10023	FBgn0020440
Fer (-)	FER tyrosine kinase	CG8874	FBgn0000723
Hop	hopscotch	CG1594	FBgn0004864
htl	heartless	CG7223	FBgn0010389
InR	Insulin-like receptor	CG18402	FBgn0283499
Nrk (-)	Neurospecific receptor kinase	CG4007	FBgn0020391
Otk (-)	Off-track	CG8967	FBgn0004839
Pvr	PDGF- and VEGF-receptor related	CG8222	FBgn0032006
Ret (-)	Ret oncogene	CG14396	FBgn0011829
Ror (-)	Ror	CG4926	FBgn0010407
Sev (-)	sevenless	CG18085	FBgn0003366
Shark	SH2 ankyrin repeat kinase	CG18247	FBgn0015295
Src42A	Src oncogene at 42A	CG44128	FBgn0264959
Src64B	Src oncogene at 64B	CG7524	FBgn0262733
Tie	Tie-like receptor tyrosine kinase	CG7525	FBgn0014073
tor	torso	CG1389	FBgn0003733
Wee1	Wee1 kinase	CG4488	FBgn0011737
Wsck	Wsck	CG31127	FBgn0046685

Table 2**Tyrosine kinase RNAi lines used for UAS-Pez cross.**

Kinase	Stock number	Location
Abl	35327	BL
Abl	61170	BL
Ack	33899	BL
Csk	41712	BL
Shark	25788	BL
Src42A	55868	BL
Src42A	44039/02755	BL
Src42A	100708/108017	Vienna
Src42A	17643/8338	Vienna
Src42A	26019 (on 3)	Vienna
Src42A	35990	BL
Src64B	30517	BL

CHAPTER III: TOXICITY OF A FLYLIGHT GAL4 DRIVER

Introduction

In *Drosophila*, the ability to spatially control gene expression allows for a broad range of studies. One effective method to manipulate gene expression is through the GAL4/UAS system (Fischer et al. 1988; Brand and Perrimon, 1993). In this system, the yeast transcription activator GAL4 can be expressed under the control of a *cis*-regulatory element (CRE) to act as a "driver" of gene expression in specific tissue. The upstream activation sequence (UAS) is the enhancer bound by GAL4 which recruits transcription factors to induce expression of downstream genes. The GAL4/UAS expression system is especially useful for misexpression studies as yeast GAL4 generally has little or no effect alone and UAS is absent or innocuous in *Drosophila*.

The *Drosophila scalloped* (*sd*) gene plays a role in wing development. Scalloped is a Transcription Enhancer Factor-1/ AbaA Domain (TEAD) family DNA binding protein, first characterized by its mutant wing phenotype in 1929 (Gruneberg 1929; Campbell et al. 1992). Along with co-factors *vestigial* (*vg*) and *yorkie* (*yki*), Sd functions as a DNA sequence-specific transcription factor controlling cell proliferation in the developing wing (Williams et al. 1991). When associated with Yki, Sd acts to promote growth and proliferation via the Hippo pathway to affect multiple aspects in wing morphogenesis (Guss et al. 2001; Bandura and Edgar, 2008). Scalloped activity is also present in neuronal and muscle cells, where it assists in neuronal pathfinding (Guss et al. 2013; Corty et al. 2016).

A collection of GAL4 driver lines generated in the FlyLight Project at the Janelia Research Campus have been characterized by immunohistochemistry and computationally assigned to regions of the *Drosophila* brain (Pfeiffer et al. 2008; Jenett et al. 2012; Jory et al. 2012). Enhancer regions of 1,200 genes involved in the *Drosophila* brain were cloned with

GAL4 coding region into a vector and then inserted into the fly genome (Pfeiffer et al. 2008). Insertion of these putative enhancer regions were mediated by a site-specific *attP2 PhiC31* recombinase resulting in each enhancer inserted at the same genomic location, allowing for reliable comparison of expression patterns. (Groth et al. 2004; Pfeiffer et al. 2008; Jenett et al. 2012). This technique generated over 6,500 GAL4-driver lines including a line containing an enhancer/CRE region from approximately 2,200 bp of the first intronic region of *sd*, *sd*^{R94B03}-*GAL4* (w[1118]; P{y[+t7.7] w[+mC]=GMR94B03-GAL4}attP2 flybase.org/reports/FBti0139744)

Interestingly, *sd*^{R94B03}-*GAL4* displays a ‘scalloping’ phenotype at the edge of the adult wings even in the absence of a UAS associated gene element. GAL4-associated phenotypes have been previously reported in *Drosophila*, potentially due to a stress or immune response to high levels of GAL4 production (Kramer and Staveley, 2003; Bollepalli et al. 2017; Liu and Lehmann 2008). This wing phenotype suggests a disruption in wing morphogenesis during development by GAL4, despite expectations of no detectable phenotype when regulated by the majority of CREs.

Our preliminary work investigating *sd*^{R94B03}-*GAL4* wing scalloping has shown that caspase activity is detectable via the apoliner marker system (Edwards, unpublished) at the margins in *sd*^{R94B03}-*GAL4* pupal wing disc, indicating an increase in programmed cell death. This is a characteristic of mutations in *sd* and other wing-margin mutants. Thus, *sd*^{R94B03}-*GAL4* wing scalloping could be due to either (A) toxic effects of excessive GAL4 protein, or (B) the *sd* CRE fragment interfering with endogenous developmental genes. To further investigate the *sd*^{R94B03}-*GAL4* wing phenotype, we examined the cellular mechanism possibly responsible for an increase in caspase mediated cell death.

Results

To evaluate whether the sd^{R94B03} -*GAL4* phenotype was due to GAL4 toxicity or a non-GAL4 mechanism, we examined if GAL80ts had any influence on wing scalloping. GAL80ts is a temperature sensitive protein that is able to inhibit GAL4 activity at low temperatures (~18C), but itself becomes inactivated at higher temperature (~30C). A phenotypic reversion is seen in wings from $GAL80ts/+$; sd^{R94B03} -*GAL4*/ sd^{R94B03} -*GAL4* flies raised at ~21C compared to $+/+$; sd^{R94B03} -*GAL4*/ sd^{R94B03} -*GAL4*, and partial reversion in the same cross when raised at 25C (Fig. 1).

To determine if an increase in programmed cell death is responsible for scalloping, we examined the effects of the anti-apoptotic protein p35 in sd^{R94B03} -*GAL4* homozygotes. Inhibition of caspase activity is accomplished when cleavage of p35 itself causes a covalent binding to the caspase enzyme by p35 cleavage products to prevent further caspase activity (Bertin et al. 1996). The ectopic expression of p35 has previously been shown to partially suppress apoptosis-mediated loss of wing margin tissues (Krupp et al. 2005; Ellis et al 2018). The line, $P\{w^+, UAS-p35.H\}BHI$ was used to express p35 under control of sd^{R94B03} -*GAL4*. Wings taken from $p35/+$; sd^{R94B03} -*GAL4* / sd^{R94B03} -*GAL4* exhibit a reversion of margin scalloping, although this phenotype does not appear identical to that seen in crosses with *GAL80ts* (Fig. 1). Differences between phenotypes may be due to ectopic expression p35, as endogenous caspase activity is required for proper development of margin tissues (Krupp et al. 2005; Jafar-Nejad et al. 2006).

The wing scalloping pattern of sd^{R94B03} -*GAL4* is similar to some loss-of-function phenotypes of Notch signaling, which contributes to wing vein patterning, cell proliferation, and margin formation (de Celis and García-Bellido, 1994; Mahoney et al., 2006). To investigate if the activity of the Notch pathway was disrupted, we used the line, $P\{w^+, NRE-EGFP.S\}5A$,

which expresses GFP under the control of a Notch Response Element (NRE). Third instar $NRE/+; +/+$, $NRE/+; sd^{R94B03}-GAL4/TM6$, and $NRE/+; sd^{R94B03}-GAL4/sd^{R94B03}-GAL4$ larvae wing discs were compared for differences in GFP Notch reporter signal. Notch activity is detected at the dorsal-ventral (DV) boundary in all three crosses (Fig. 3A-C). Punctate GFP reporter punctate can be observed at the wing DV boundary in $NRE/+; sd^{R94B03}-GAL4/+$ and $NRE/+; sd^{R94B03}-GAL4/sd^{R94B03}-GAL4$ (Fig. 3B & 2C) likely resulting from the increased cell death in those genotypes.

Discussion

The GAL4/UAS binary expression system has proven to be a powerful and flexible tool for studying gene expression in *Drosophila*. While most GAL4 driver lines show no phenotype, a pronounced wing scalloping phenotype is observed in the $sd^{R94B03}-GAL4$ driver line. Observations from $p35 \times sd^{R94B03}-GAL4$ crosses indicate that scalloping is due to an increase in programmed cell death at the developing wing disc margins. GAL80ts, which binds and inactivates GAL4, produces a strong reversion of the $sd^{R94B03}-GAL4$ wing phenotype. This demonstrates that margin formation is disrupted by a UAS-independent GAL4 activity unique to this line.

However, the *sd* gene fragment in the $sd^{R94B03}-GAL4$ construct remained a possible contributor to the apoptotic effect, perhaps by affecting the regulation of local genes, or disrupting expression of the endogenous *sd* gene. Therefore, in other experiments, the $sd^{R94B03}-GAL4$ driver line was edited using the CRISPR/Cas9 system to replace the GAL4 coding region with its *Neurospora* analog QF2 to determine if the phenotypic effects are exclusive to GAL4 (Lin and Potter, 2016). Those flies have QF2 activity at the margin, but the margin is not

scalloped (not shown). Thus, GAL4 activity is sufficient to trigger a strong apoptotic response in this line.

Observations in third instar larvae wing discs indicate that Notch expression at the DV is normal in $sd^{R94B03}-GAL4/TM6$ or $sd^{R94B03}-GAL4/ sd^{R94B03}-GAL4$. Apoptosis is evident in NRE expressing cells, indicating that the GAL4 toxicity mechanism does not involve turning off Notch signaling. It remains to be determined which other pathways are impaired by GAL4. The very consistent, dosage-sensitive wing phenotype of $sd^{R94B03}-GAL4$ makes it possible to perform targeted or large-scale genetic screens that could identify this mechanism.

The yeast derived GAL4/UAS offers a convenient system for studying gene expression through its modularity. The commonly inert nature of GAL4 in most *Drosophila* cells allows for its expression while avoiding confounding phenotypes when a UAS element is absent. The wing scalloping phenotype of $sd^{R94B03}-GAL4$ driver line demonstrates the importance of evaluating newly generated driver lines, as they may still result in unexpected phenotypic side-effects.

Figure 1. Wing phenotypes of GAL80ts crossed to sd^{R94B03} -GAL4. Wings from $+/+; sd^{R94B03}$ -GAL4/ sd^{R94B03} -GAL4 raised at 21C (A), $GAL80ts/+; sd^{R94B03}$ -GAL4/ sd^{R94B03} -GAL4 raised at 21C (B), and $GAL80ts/+; sd^{R94B03}$ -GAL4/ sd^{R94B03} -GAL4 raised at 25C.

Figure 2. Wing phenotypes of UAS-p35 crossed to sd^{R94B03} -GAL4. Wings from $+/+; sd^{R94B03}$ -GAL4/ sd^{R94B03} -GAL4 (A) and $p35/+; sd^{R94B03}$ -GAL4/ sd^{R94B03} -GAL4 (B)

Figure 3. Detection of Notch GFP reporter. Wing discs from Notch GFP reporter *NRE/+; +/+* (A) *NRE/+; sd^{R94B03}/TM6* (B) and *NRE/+; sd^{R94B03}-GAL4/ sd^{R94B03}*.

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Pez

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GAL4 Toxicity

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APPENDIX A: METHODS AND MATERIALS

Isolation of total RNA from Zebrafish

Illinois State University Biosafety and IACUC protocols (04B-2011 and 15-2011) were adhered to at all times. Purchase of adult zebrafish was done through a commercial supplier. Zebrafish were euthanized by Tricaine solution (400 mg/100 ml, Sodium Bicarbonate buffered water, Western Chemical Inc.) and placed in liquid nitrogen just prior to being homogenized in 9mL guanidinium thiocyanate (TRIzol, Life Technologies). 5' RACE (Roche Life Sciences) was performed according to kit instructions. Initial reverse 5'RACE primer was designed complementary to sequence within exon 2 of the predicted *in silico pth2rb* sequence (RACE_E2_RT). 5' RACE *pth2rb* transcripts were ligated into pCDNAI/Amp and pGEMT-easy plasmids for subcloning and sequencing. Sequencing of *pth2rb* cDNA was performed at Univ. of Illinois Urbana-Champaign Core Sequencing Facility. Full length *pth2rb* was amplified by PCR from 1st strand RT-PCR synthesized cDNA (from primer Pth2rb_3UTR_RT) with primers at the 5' UTR of the 5' RACE sequence (Pth2rb_5UTR_3) and complementary to the *pth2rb* 3'UTR (Pth2rb_3UTR_2). Full-length *pth2rb* was ligated into pCDNAI/Amp and pGEMT-easy.

Primers:

Pth2rb_3UTR_RT: TCCAGTTTCAATCATGAACGTATCC

RACE_E2_RT: CGCAGCATGAACGACACAAATAG

Pth2rb_5UTR_3: AGCTGGATAAACCCACCGGA

Pth2rb_3UTR_2: GTCAGGTTTTTTTAAAGTACTTCCTC

Sequence analysis.

All six PTH receptor homologs from human and *Danio rerio* were identified by BLAST. Multi-alignment was made using PSI/TM-Coffee (<http://tcoffee.crg.cat>; Floden et al. 2016), then manually edited to reduce weakly-supported gaps. Sequence files aligned: NP_000307; NP_571432; NP_571453; NP_005039; NP_571452; XP_017208978. Figure made by inputting

alignment to Multiple Align Show at The Sequence Manipulation Suite (bioinformatics.org), using this set of similarity groups: (FILMVW) (KR) (DE) (ST). Output was annotated manually in Photoshop. The structure figures (4-5) were made from PDB 6FJ3 using CCP4 Molecular Graphics Program v2.10.10 (www.ccp4.ac.uk; Winn et al. 2011).

***In situ* hybridization**

Embryos of various stages were cryosectioned and sections were used for in situ hybridization as described (Rodríguez-Marí et al. 2005). Probe synthesis and whole mount in situ hybridization analyses were performed as previously described by (Jowett & Yan, 1996). Complementary RNA (cRNA) probes were produced by using the following restriction enzymes and RNA polymerases: antisense *pth2ra*, *Bam*HI and SP6; antisense *pth2*, *Sph*I and SP6. *Pth2rb* in pCDNAI/AMP was linearized with *Bam*HI prior to cRNA synthesis with SP6 polymerase.

Cell transfection and PTHR plasmids

An HEK-293 cell line (GS-22A) that stably expresses the luciferase-based pGloSensor-22F (GloSensor) cAMP reporter plasmid (Promega Corp. USA) was used for cAMP ligand-dose-response analyses. The cells were seeded in white 96-well plates and, when 80% confluent, were transfected using Fugene-6 reagent with plasmid DNA encoding either the human PTH1R (hPTH1R), human PTH2R (hPTH2R), zebrafish *Pth2ra* (zPth2ra) or zebrafish *Pthr2b* (zPth2rb) and 80 ng DNA per well. The plasmid vector used for expressing hPTH1R and hPTH2R was pCDNA3.1(+).

GloSensor assays of cAMP signaling

Two days after transfection when the GS-22A cells were confluent, the media was replaced with CO₂-independent medium containing BSA (0.1%, Sigma) and luciferin (0.5 mM, Biotium); and incubated for 20 minutes at room temperature. Then test ligands at varying

concentrations were added and incubations continued for an additional period of up to 90 minutes, during which time changes in luminescence arising from intracellular cAMP binding to the GloSensor reporter protein were measured at two-minute intervals using a PerkinElmer Envision plate reader. Ligand dose-response curves were generated using the peak luminescence response measured for each well/ligand concentration, which typically occurred ~20 minutes after ligand addition.

cAMP data calculations

Data were processed using Excel 2008 (Microsoft Corp. Redmond, WA) and Prism 5.0 (GraphPad Software Inc., La Jolla, CA). Aggregate data are expressed as means $\pm$ S.E.M. Curves were fit to the data using a four-parameter, non-linear regression function. Statistical analyses were performed using the Student's t-test (two-tailed, unequal variances), significance was inferred from P values of less than 0.05.

Pez crosses and confocal analysis

Pez overexpression marker line 'UAS-Pez':

[*Hsp70-FLP*], [*Act5C>CD2>GAL4*]; [*GMR-GAL4*], [UAS-Pez]; [UAS-mCD8-mCherry]

UAS-Pez marker line (males) was crossed with virgin females from RNAi stocks. These flies carry an *Act5C>CD2>GAL4* construct; heat shock induction of FLP recombinase triggers excision of the *CD2* stuffer fragment, allowing high-level GAL4 production from the *Act5C* promoter in random clones of cells. Before ovary dissection, 5-8 females were transferred to a new tube with fresh yeast and males to stimulate egg production. Flies were heat shocked for 50 mins at 38C and then transferred to a 25C chamber for 2.5 days. Ovaries were dissected in 1x PBS and immediately fixed for 15 min in 2% Formaldehyde. Ovaries were washed in 1x PBS 3-4 times and then transferred to 1x PBT (PBS + Triton 0.2%). Tissues were

blocked with PBT +4% goat serum for 45 mins at room temperature. Primary antibodies (1:1000 dil) were added to ovaries overnight at 4C or 5hr at room temperature in PBT + 1% goat serum (PBTG) (Singari et al. 2014; Pignoni and Zipursky 1997). Ovaries were washed 3-4 times with PBTG before incubation with secondary antibodies overnight at 4C. Ovaries were washed with PBTG and mounted on slides in vectashield.

***sd*^{R94B03}-*GAL4* wing disc dissection**

Wing discs were dissected from 3rd instar larvae in 1x PBS and immediately fixed for 15 min in 2% Formaldehyde. Wing discs were then mounted in vectashield prior to imaging by fluorescent microscopy to detect GFP.

Contributing work in:

Dale, M. D., Mortimer, E. M., Kolli, S., Achramowicz, E., Borchert, G., Juliano, S. A., ... Rubin, D. A. (2015). Bone-Remodeling Transcript Levels Are Independent of Perching in End-of-Lay White Leghorn Chickens. *International Journal of Molecular Sciences*, 16(2), 2663–2677.

Cloning cDNA for Sequence Validation:

All amplicons generated by qPCR were electrophoresed, excised, ligated into pGEMT-easy (Promega), transformed into TOP 10 *E. coli* cells (Invitrogen, Carlsbad, CA, USA), screened and sequenced with T7 and Sp6 primers, as previously described (Yan et al. 2012). The subcloning and DNA sequencing was performed to validate the amplicons generated. Using this information, final validated primer pairs were subsequently selected for each locus.

APPENDIX B: SUPPLEMENTARY TABLES & FIGURES

Abbreviation	Full gene name	CG number	FlyBase record #
Abl	Abl tyrosine kinase	CG4032	FBgn0000017
Ack	Activated Cdc42 kinase	CG14992	FBgn0028484
Ack-like	Activated Cdc42 kinase-like	CG43741	FBgn0263998
Alk	Anaplastic lymphoma kinase	CG8250	FBgn0040505
Btk29A	Btk family kinase at 29A	CG8049	FBgn0003502
Btl	Breathless	CG32134	FBgn0285896
Cad96Ca	Cadherin 96Ca	CG10244	FBgn0022800
CG3277	CG3277	CG3277	FBgn0031518
Csk	C-terminal Src kinase	CG42317	FBgn0262081
Ddr	Discoidin domain receptor	CG33531	FBgn0053531
dnt	Doughnut on 2	CG17559	FBgn0024245
drl	derailed	CG17348	FBgn0015380
Drl-2	Derailed 2	CG3915	FBgn0033791
Egfr	Epidermal growth factor receptor	CG10079	FBgn0003731
Eph	Eph receptor tyrosine kinase	CG1511	FBgn0025936
Fak	Focal adhesion kinase	CG10023	FBgn0020440
Fer	FER tyrosine kinase	CG8874	FBgn0000723
Hop	hopscotch	CG1594	FBgn0004864
htl	heartless	CG7223	FBgn0010389
InR	Insulin-like receptor	CG18402	FBgn0283499
Nrk	Neurospecific receptor kinase	CG4007	FBgn0020391
otk	Off-track	CG8967	FBgn0004839
Pvr	PDGF- and VEGF-receptor related	CG8222	FBgn0032006
Ret	Ret oncogene	CG14396	FBgn0011829
Ror	Ror	CG4926	FBgn0010407
sev	sevenless	CG18085	FBgn0003366
Shark	SH2 ankyrin repeat kinase	CG18247	FBgn0015295
Src42A	Src oncogene at 42A	CG44128	FBgn0264959
Src64B	Src oncogene at 64B	CG7524	FBgn0262733
Tie	Tie-like receptor tyrosine kinase	CG7525	FBgn0014073
tor	torso	CG1389	FBgn0003733
Wee1	Wee1 kinase	CG4488	FBgn0011737
Wsck	Wsck	CG31127	FBgn0046685

Supplemental Table 1. Comprehensive list of candidate cytoplasmic tyrosine kinases for RNAi

knockdown in UAS-Pez. (*) indicates CTK knockdowns described in this this work.

Supplemental Figure 1. Map of *Src42A* targeting RNAi lines crossed to UAS-Pez. *Src42A* TRiP RNAi knockdown lines targeting *Src42A-A* (Blue stars), *Src42A-B* (Red Star), or both forms via their conserved C-terminus of *Src42A-A/-B* (Green star).

Supplemental Figure 2. UAS-Pez crossed to *Src42A* RNAi (55868). Additional confocal images of clonal follicle cells overexpressing Pez and *Src42A* RNAi (55868) knockdown. Pez visible in overlay as red, pTyr in green, and mCD8-RFP clone marker in blue.