Rare hypomorphic human variation in the heptahelical domain of SMOOTHENED contributes to holoprosencephaly phenotypes

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Abstract

Holoprosencephaly (HPE) is the most common congenital anomaly affecting the forebrain and face in humans and occurs as frequently as 1:250 conceptions or 1:10,000 livebirths. Sonic hedgehog (SHH) is one of the best characterized HPE genes that plays crucial roles in numerous developmental processes including midline neural patterning and craniofacial development. The Frizzled class G-Protein Coupled Receptor (GPCR) SMOOTHENED (SMO), whose signalling activity is tightly regulated, is the sole obligate transducer of hedgehog-related signals. However, except for previous reports of somatic oncogenic driver mutations in human cancers (or mosaic tumors in rare syndromes), any potential disease-related role of SMO genetic variation in humans is largely unknown. To our knowledge, ours is the first report of a human hypomorphic variant revealed by functional testing of seven distinct non-synonymous SMO variants derived from HPE molecular and clinical data. Here we describe several zebrafish bioassays developed and guided by a systems biology analysis. This analysis strategy, and detection of hypomorphic variation in human SMO, demonstrates the necessity of integrating the genomic variant findings in HPE probands with other components of the hedgehog gene regulatory network (GRN) in overall medical interpretations.

INTRODUCTION

Holoprosencephaly (HPE) is understood to be a failure in the generation of midline signals that normally instruct eye field and forebrain division (Roessler et al., 1996; Belloni et al., 1996; Chiang et al., 1996; Abramyan 2019). Five principal genes: *SHH, ZIC2, SIX3* ,*FGF8* and *FGFR1* , are the most commonly detected genetic factors responsible for HPE (Roessler et al., 2018b). While *de novo FGFR1* mutations match a specific syndromic set of brain and limb findings, called Hartsfield syndrome (Simonis et al., 2013; Hong et al., 2016), mutations in the first four HPE genes fit into a more typical non-syndromic pattern in probands with a normal chromosome analysis and microarray (Brown et al., 1998; Wallis et al., 1999; Hong et al., 2018; Roessler and Muenke, 2010). Each of these five driver genes is required in a conserved developmental program during gastrulation for midline specification. The roles of several infrequent minor genes, e.g. *PTCH1, DISP1, GAS1, TGIF1, DLL1* , etc. are less well understood (Roessler et al., 2018a). However, a common pathogenetic HPE mechanism leading to diminished midline hedgehog signaling links the major driver mutations into a conserved gene regulatory network (GRN, Suppl. Fig.1).

Hedgehog (Hh) proteins secreted from the vertebrate prechordal plate and ventral diencephalic midline are lipid-modified morphogens that play a central role in patterning the early forebrain primordium (Ohkubo et al., 2002; Cooper et al., 2003; Wilson and Houart, 2004; Bertrand and Dahmane, 2006; Storm et al., 2006; Blassberg et al., 2016). Shh functions by binding and inactivating Patched (e.g. human *PTCH1* ,*PTCH2*

), the major sterol-sensing and sterol-transporter related receptor(s) for Hh ligands, which constitutively and non-stoichiometrically suppress Smo activity by depriving this GPCR receptor of cholesterol and/or related sterol ligands needed to stabilize its active conformation (Taipale et al., 2002; Ingham et al., 2011; Briscoe and Therond, 2013; Luchetti et al., 2016; Huang et al., 2018; Hu and Song, 2019; Deshpande et al., 2019). *SMO* activates a classical signal cascade that ultimately regulates the post-translational state of Gli family transcription factors, which in turn bind enhancers of target genes (Huangfu and Anderson, 2006). Hh morphogen binding to Patched decreases its presence in the cilium leading to its internalization and degradation. Activation of Smo in vertebrates is correlated with its enrichment in the cilium (Huangfu and Anderson, 2005; Corbit et al., 2005; Gigante et al., 2018) as well as ultimately altering the processing of Gli transcription factors from truncated repressors to full-length activators. The role of non-classical GPCR signaling via *SMO* in human pathologies is less clearly understood (Arensdorf et al., 2016; Qi et al., 2019). However, the heptahelical domain is the frequent target for recurrent activating mutations in cancer and somatic tumors (Xie et al., 1998; Lam et al., 1999; Taipale et al., 2000; Ayers and Therond, 2010; Twigg et al., 2016), while variation within the remaining domains characteristic of the GPCR superfamily are less frequently observed (see Figure 1). Mutagenesis screens in *Drosophila* and zebrafish have clearly demonstrated that there is a functional consequence to variation in, and cooperative interactions between, all of these domains (Nakano et al., 2004; Zhao et al., 2007; Aanstad et al., 2009; Nachtergaele et al., 2013). Furthermore, recent structural studies of various vertebrate Smo molecules emphasize that both agonist and antagonist binding can alter the three-dimensional structure(s) of Smo orthologs and shift the orientation of all constituent domains (Byrne et al., 2016; Luchetti et al., 2016; Huang et al., 2016; Huang et al., 2018; Byrne et al., 2018; Zhang et al., 2018). Therefore, for completeness we chose to analyze all variants detected in our series of HPE probands.

SHH signaling regulates the transcription of genes involved in the specification of the ventral forebrain primordium, the resolution of a primordial single eye field into paired optic vesicles, as well as optic nerve and retinal development (Li et al., 1997; England et al., 2006; Sanek et al., 2009; Xavier et al., 2016). This early function of*SHH* in the splitting of the eye field into two lateral optic primordia followed by induction of optic stalk tissue at the expense of neural retina is key to our understanding of HPE and the dramatic cyclopic phenotypes seen in both animal models and humans (Chiang et al., 1996; Hammerschmidt et al., 1996; Hammerschmidt and McMahon, 1998; Vinothkumar et al., 2008; Pillai-Kastoori et al., 2015). Our understanding of these morphological and gene expression changes influenced our approach to *SMO* bioassay development.

Here we report our functional testing in zebrafish of seven non-synonymous variants of human *SMO* derived from HPE molecular genomic and clinical data. Our studies reveal some of the inherent difficulties of *in vivo* bioassay systems that depend on a lag in experimental intervention (e.g. injection of the *SMO* gene) and measurements of biological responses (e.g. marker gene changes, or morphogenetic phenotypes). Given the complex GRN involved in hedgehog signaling and its feedback wiring we now demonstrate that investigators need to incorporate a clear understanding of these regulatory relationships in assay development as well as in medical genetic interpretation.

MATERIALS AND METHODS

Patient samples and informed consent

All subjects provided written informed consent for themselves and their affected children using approved forms and oversight from our NIH, NHGRI Institutional Review Board. Institutional approval has been obtained for data sharing with investigators with Institutional Review Board protocols studying brain development and HPE through web application to dbGAP.

Zebrafish husbandry

The wildtype-TAB5 strain was used in this study. The tropical zebrafish were maintained at 28 according to "The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (*Danio rerio*)" in our zebrafish facility at NIH. All experimental procedures have been approved and performed by following procedures described

in our National Human Genome Research Institute animal protocol at the National Institutes of Health.

Human cDNA constructs

The wild-type version of human *SMO* (NM_601500) full length cDNA clone was purchased from OriGene (SC122724, OriGene Technologies) and subcloned into the mammalian expression vector pCS2+. Human *SMO*point mutation constructs were generated by PCR allowing for the modification of each patient-associated single base alteration. All human *SMO* point mutation variant constructs were generated using Site-Directed Mutagenesis kit (QuickChange, Agilent Technologies) according to the manufactures' instructions without modification. All subcloned cDNA constructs were verified by Sanger sequencing.

Morpholinos and RNA injections

The effect of zebrafish *patched1* and *patched2* morphants has been previous reported (Koudijs et al., 2008) and, therefore, we ordered Morpholino Oligonucleotides (MO) targeting each translation start site of zebrafish *patched1* and *patched2* mRNA, as well as control MOs, were developed by Gene Tools (Gene Tools LLC) and designed as follows: the *patched1* translation-blocking MO (ATG MO), 5'-AGGAGACATTAACAGCCGAGGCCAT-3' and the *patched2* ATG MO, 5'-CATAGTCCAAACGGGAGGCAGAAGA-3'. MOs were diluted in RNAse-free water to 0.1-0.5 ng/nl (*ptch* 1, *ptch* 2), 5 ng/nl (con MO).

In vitro **transcription and mRNA injections**

To generate mRNA, WT and mutant forms of *SMO* cDNA were generated using the mMESSAGE mMACHINE SP6 transcription kit (Thermo Fisher Scientific). For the *SMO* mRNA over-expression experiments, we used a 200 pg bolus of *SMO* synthetic mRNA with a 2 pg bolus of*SHH* . For the rescue experiments using our K/O model, we used a 80 pg bolus of *SMO* synthetic mRNA. For the K/D assay, we used a 100 pg bolus of *SMO* synthetic mRNA with 0.1 ng bolus of both*ptch1* and *ptch2* MO. Each injection cocktail was injected into fertilized one cell stage embryos. Either a gfp or control MO was used as a negative control for over-expression and K/O experiments or K/D experiment, respectively. Trials of appropriate dose-response were performed before each experiment (Suppl.Fig.2 and Suppl.Tables S1,4-6). All experimental tests of mRNA injections in this study were performed with 50-80 embryos/injection, in triplicate and on different days.

Generation of *smo* mutant fish and genotyping

A single guide RNA (sgRNA) targeting exon 5 of *smo* (GenBank accession number: NM_131027) (GGCTGATGGGTGGTGCCAA) was designed using the 'ZebrafishGenomics"' track on the UCSC Genome Browser. Primers were designed to amplify a 275 bp region flanking the target site for use in all screening and subsequent genotyping steps using the previously described fluorescent PCR assays (Sood et al. 2013). The primer sequences are as follows: *smo* -E5-Fwd (5'- TGTAAAACGACGGCCAGTTTACTCGCTTATGTCTGGAG) and *smo* -E5-Rev (5'- GTGTCTTTAACTGAATCTGCATCCACC). Synthesis of target oligonucleotide (Integrated DNA Technologies), preparation of mRNA, microinjection, evaluation of sgRNA activity by CRISPR-STAT and generation of mutant lines were carried out as described previously (Varshney et al., 2016). Briefly, Wildtype embryos (TAB5) were injected with sgRNAs (5pg) and Cas9 mRNA (300pg) and grown to adulthood. Screening for germline transmission of indels was carried out by analysis of 8 embryos from the progeny of each founder fish at 24 hours post fertilization by fluorescent PCR. Progeny of founder fish with desired mutant alleles were grown to adulthood and genotyped to identify heterozygous fish for subsequent phenotype analysis. The mutant alleles were sequenced to determine their predicted effect on the encoded protein.

In situ **hybridization**

Developmentally staged zebrafish (13, 18 and 24 hpf) embryos were fixed with 4% paraformaldehyde (Sigma) during an over-night incubation at 4. Whole-mount *in situ* hybridization was performed using the conventional alkaline-phosphatase-based single-color method (Kudoh et al., 2001). We used a gene paired box 2a (*pax2a*) (Sanek et al., 2009; Lee et al., 2008) and paired box 6a (*pax6a*) (Sanek et al., 2009; Lee et al.,

2008) at 18 and 24 hpf, neuronal differentiation 4 (*neurod4* ; previously known as *zath3*) (Miyake et al., 2012) at 24 hpf.

Zebrafish assessment

Live embryo phenotypes were analyzed using a Leica MZ12.5 at 24 and 48hpf. The pattern of rescue was determined by the somite shape and eye color in the rescue experiment. We determined the phenotypic difference in rescue experiments based on specific abnormal features of *smo*K/O model: *U* -type tail and less than half area of retina pigmentation in the eye region. Embryo phenotypes with partial rescue of eye shown over half area of retina pigmentation. Embryo phenotypes with partial rescue of somite shown not curled *U* -type tail but rather an unstraight tail. Live embryo images of lateral or dorso-ventral views, images of gene expression after *in situ* hybridization and images for eye area or somite analysis were acquired on a ZEISS AxioCam HRC Camera and AxioVision SE64 software after embedded embryos in 3% Methylcellulose (Sigma): Live embryo and eye area or somite analysis; in Glycerol (Invitrogen): *in situ* hybridization. Somite angle measurements were taken at the midpoint between the proximal hindgut and the anus at 24hpf embryos using ImageJ software. For eye area measurements, lateral images at 24hpf embryos were quantified using ImageJ software. For eye and somite analysis, total 45 embryos/group: 15 embryos/group in each triplicate injection, were analyzed.

Statistical analysis

All RNA injections are performed on different days and in three independent injections. Results are expressed as means±SD. For statistical analysis, experimental data was calculated with Student's t-test using JMP 13 (SAS Institute Inc).

RESULTS

SMO mutation findings in HPE patients

We recently reported the results of a Targeted Capture analysis of 153 developmental genes (and a similar number of conserved non-coding elements) among 333 HPE probands in an effort to identify both driver mutations and potential modifiers (Roessler et al., 2018a). The*SMO* gene was identified as a potential candidate gene based on the identification of six families with gene variants of undetermined significance. As described in Table 1, two of these families had a detectable driver mutation with a co-morbid variant in *SMO* (see summary Suppl. TableS7, and annotations for each proband Tables S8-13). In order to better understand the potential roles of these variants we developed functional studies.

Proband #1 has a maternal *FGF8* driver mutation yet also inherited a complex *SMO* variant with two variants of uncertain significant (VOUS, *in cis*) from the father (Hong et al., 2018). We chose to investigate both *SMO* variants individually to better determine the potential for digenic inheritance in this case. Similarly, although proband #6 has a typical *de novo* driver mutation in*SHH* , we chose to directly examine the predicted "likely benign" predictions for the co-inherited *SMO* variant. Our working hypothesis continues to be that modifier genes need not meet the same guideline considerations as more conspicuous driver mutations with respect to allele frequency (Hong et al., 2017; Roessler et al., 2018a; Roessler et al., 2018b).

Probands #2 and #3 present with findings that appear to reflect common benign *SMO* variants inherited from otherwise healthy parents. In neither case are the potentially co-morbid alterations predicted to be functionally abnormal. In the case of proband #3, almost all of the co-variants were inherited from the healthy father suggesting that even if they had synergistic effects it would raise the question of why the father was not similarly affected. Nevertheless, we included these*SMO* variants as likely negative controls.

Probands #4 represents a similar case of exclusive maternal inheritance of the potentially interacting loci. Although the *SMO* variant is common in the general population it is predicted to be deleterious. Thus, since we classified it as a rare VOUS we felt compelled to try to determine its biological activity.

Perhaps the most interesting *SMO* variant was identified in proband #5 as an initially novel alteration of p.V404M. Later it was identified as a single detection in the gnomad data base. Its rarity and location in the

heptahelical domain suggested that of the seven variants identified in HPE probands this missense alteration deserved closer examination in functional studies.

Domain structure of human SMO and variant annotation

The *SMO* transcript encodes a typical domain structure of a GPCR-like signal transducer (Fig.1A, 1B) that traffics from intracellular pools to the plasma membrane and ultimately through the cilium following ligand stimulation. A key feature of *SMO* is its heptahelical transmembrane domain, that is the site of natural and synthetic agonist and antagonist binding as well as activating mutations in human cancers. The seven missense variants detected among HPE probands are described in Table 1 and their positions are annotated in Fig.1B. The primary amino acid comparison data indicates that the mutant residues p.R113, p.R199, p.V404, p.R772 are largely evolutionarily conserved (Fig.1C). However, the potential consequences of the variations are difficult to predict.

Gain-of-function approach: Over-expression analysis of*SMO* variants

To experimentally analyze the effect of these point mutation changes, we conducted over-expression experiments as our first approach. We microinjected wild type (WT) human *SMO* mRNA to determine the appropriate dose which would induce an abnormal phenotype in WT zebrafish (TAB5). However, in the pilot experiments using 100pg to 600pg*SMO* mRNA we did not readily induce abnormal phenotypes (Suppl. Fig.2). Based on our understanding of the hedgehog GRN described in Suppl.Fig.1 we hypothesized that the microinjected *SMO* was inhibited by *ptch* in a negative feed-back loop attenuating the biological response.

Optimization of the biological response to *SMO*over-expression

To minimize the inhibition of exogenous *SMO* via endogenous zebrafish *ptch* , we co-injected a small amount of WT human*SHH* mRNA with *SMO* mRNA. We identified an optimal combination to induce abnormal phenotypes is the co-injection of 2pg of*SHH* mRNA and 200pg *SMO* mRNA (see the dose-response pilot study, Suppl.Fig.2). We could independently demonstrate that 2pg of*SHH* mRNA (alone) or 200pg of *SMO* mRNA (alone) has minimal phenotypic consequences that were similar to our *gfp* control mRNA-injected embryos (Suppl.TableS1). We could demonstrate that co-injection induced a striking synergistic effect for embryos that was consistent with our understanding of the Hh GRN. The majority of co-injected embryos resulted in decreased pigmentation of the retina at 24 to 48 hours post fertilization (hpf). Cyclopia as a severe phenotype was observed in a few embryos at 24hpf (Fig.2A, B).

SHH/SMO **co-expression approach to the variant analysis**

As a first step to quantify the biological consequences of each*SMO* variant, we utilized this optimal co-injection dose (Suppl.Fig.2). The percentage of abnormal phenotypes, based on their gross microscopic appearance, between each of the seven variants indicated very mild allelic differences (Fig.2B; Suppl.TableS2 and S3). Indeed, these variants were similar in bioactivity to WT *SMO*under identical conditions.

To investigate any difference in biological activities between variants, we performed *in situ* hybridization at 18 to 24hpf using zebrafish*pax2a* and *pax6a* , as eye developmental markers, and*neurod4* as a neural marker. The expression level of *pax6a*(Fig.2C, D) and *neurod4* (Fig.2E, F) around the eye region showed that all variants have a Type II phenotype with suppression of each genes' expression at 19hpf (*pax6a*) or 24hpf (*neurod4*) (Suppl.Table3). The *pax2a* up-regulation (Fig.2G, H), a known antagonist of *pax6a* , expression pattern showed that all variants have Type II phenotype with enhancement of *pax2a*expression at 24hpf (Suppl.Table3). In all markers shown here, the percent of embryos with Type II expression patterns were over 80% among all variants tested under these conditions. Although we can clearly demonstrate the expected marker changes, our ability to measure subtle differences between alleles was limited by the near saturating dose required to elicit these changes. We conclude that none of the HPE variants are complete loss-of-function under these test conditions. Furthermore, despite minor differences between variant alleles these changes in gene expression patterns are qualitatively distinct, but not at a statistically significant level.

Loss-of-function approach: Analysis of *SMO* variants using a Zebrafish Knock Out (K/O)

model rescue assay

To better understand the function of these *SMO* variants we performed rescue experiments involving the injection of human *SMO*transcripts and measuring the biological activity of each variant on developing zebrafish embryos using a CRISPR/Cas9 genetic mutant in this study. We confirmed that the vast majority of *smo-/smo*-homozygous mutants (Fig.3B) manifested a shortened body trunk, curled*U* -type tail, heart edema, decreased eye color of the retina, and microphthalmia (Chen et al., 2001). We collected *smo-/smo*-homozygous mutants by mating smo^{+}/smo - breeding pairs. We optimized this assay by empirically determining the most effective dose for phenotypic rescue using the WT human *SMO* . We found our ideal assay conditions to be 80pg of human WT *SMO* mRNA based on the observed trunk-tail shape and eye color recovery (Suppl.Table4). There were three types of phenotypic rescue: complete rescue (Fig.3*B* _Control and TypeI), or partial rescue, scored as primarily eye (Fig.3*B* _TypeII) or primarily body rescue (Fig.3*B* _TypeIII). The genotype was assessed by fluorescence PCR after phenotype confirmation. The abnormal phenotype of *smo-/smo*-homozygous is efficiently rescued by microinjection of WT *SMO* and all variants at 48hpf except for p.V404M (Fig.3C). Genotyped embryos showed that p.V404M was significantly deficient in its' ability to rescue the *smo-/smo-* phenotype. Furthermore, the proportion of rescue types showed the percentage of partial rescue, combined percentages of eye rescue and body rescue, is significantly different only with the p.V404M variant (Fig.3D). Essentially identical results for the 7 variants were obtained at 24hpf by scoring for simple complete rescue using a much lower 10pg dose. However, the mutant phenotype began to re-appear at later time points (data not shown). Interestingly, we note that Type III rescue was not detected with p.R113Q.

The Knock-Down (K/D) of patched: *ptch* MO experiments

We hypothesized that the synergy between low dose *SHH* and*SMO* reflected an attenuation of the negative inhibition exerted by endogenous zebrafish *ptch* . We chose p.V404M as representative of a hypo-rescued allele (see above) and evaluated three of the variants that have a mutation in each of the other *SMO* domains as comparative alleles. Morpholinos (MO) targeting *ptch1* or*ptch2* were used to minimize the effect of zebrafish *ptch*negative feedback inhibition towards exogenous *SMO* . The injection of low doses of *ptch1* 0.1ng and *ptch2* 0.1ng, or when either is injected separately, resulted in normal phenotypes (Suppl.TableS5). However, we also observed that MO doses over 0.25 ng of*ptch1* or 0.2 ng of *ptch2* in single injection experiments leads to observable phenotypes, especially microphthalmia and slightly curved trunk (data not shown). We found an optimal assay condition to be 100 pg of human WT *SMO* mRNA and the combination of 0.1 ng of each*ptch1* MO and *ptch2* MO based on the observed microphthalmia and trunk shape abnormality (data not shown).

Thereafter, this optimized combination injection was performed using each of the four domain-representative variant *SMO* mRNAs. The results show that the eye area of embryos injected with *gfp* are significantly larger than that of others (P <0.0001). WT*SMO* produced the expected micropthalmia at the test dose. The eye area of embryos injected with p.V404M is significantly smaller than that of other variants (Fig.4A*, a* and *b*), suggesting that the p.R113Q, p.R199W, and p.P687L point mutations affect eye development less than p.V404M point mutation. However, these findings are not substantially different from the WT control.

Our measurement of the trunk angle shows that embryos injected with WT (or all four tested variants) are significantly wider than that of*gfp* (P <0.0001), a known property of midline Hh signaling on somite development. In comparisons between the four variants, p.R113Q has a significantly smaller somite angle than that of p.R199W, p.P687L and *gfp* (Fig.4B *a'* and *b'*), suggesting that the p.R113Q mutation is less bioactive under these assay conditions. Taken together, although these biometric measures were more readily quantifiable, these results do not provide unambiguous evidence for either gain or loss-of-function when directly compared to the WT control.

DISCUSSION

Precision medicine utilizing genomic methods implies that medical geneticists can effectively navigate a series complex interactions that ultimately induce malformation conditions such as HPE. The challenges to this

effort are many. These can include, in part, the variable quality of the data, the impulse to over-filter datasets in an effort to simplify the genetic interpretation, the lack of clear criteria for driver mutations as either sole or multifactorial factors (e.g. digenic inheritance), the paucity of models for determining modifier effects, the frequent lack of clinical details of the family structure and affection status, any teratogen/environmental exposure considerations, the incomplete understanding of potential gene:gene and/or gene:environment interactions, the lack of effective guidelines for non-coding variation, the tendency to ignore the key role of the timing of these interacting factors, as well as the continuously evolving considerations attempting to foster an integrated interpretation into medical practice. Extrapolating from lessons gleaned from the example of carcinogenesis clearly supports the role of considering both genes and the environment. This is almost certainly the case with the pathogenesis of HPE. Furthermore, the genetic variation of human *SMO* is now clearly implicated in both rare developmental malformations, as well as somatic variants in human cancer. We now argue that the knowledge of the Hh pathway components with respect to their fundamental sequence conservation and their gene regulatory relationships provides insight into both.

Herein, we investigated the role of the *SMO* gene in craniofacial and forebrain development using zebrafish as a model system. This study is intended as a comprehensive analysis of variants detected in a malformation (HPE) known to be caused by disturbed function of the hedgehog pathway. In doing so, our study directly addresses the apparent contradiction that although impaired Hh signaling is the most common pathological mechanism for HPE, the hypo-function of *SMO* is indeed uncommon. And yet, gain-of-function mutations in *SMO* are a frequent cause of both basal cell carcinoma and many of the most common pediatric brain cancers.

Many of the complicating factors in precision medicine are evident in the present report on these six cases. Current guidelines (Richards et al., 2015) appropriately rank the relative importance of the novelty of a variant, the *de novo* status, the co-inheritance of relevant variants, instances of similar variants with proven pathogenicity, the family history, similarly affected relatives, the bioinformatic pathogenicity scores, etc., into an integrated determination. Of the proband-specific findings in this report (Suppl.TableS7), only the*FGF8* and *SHH* mutations meet these criteria for pathogenicity, while the *SMO* variants remained unclassified. Thus, functional studies are often the determinative factor in assigning clarity to the interpretation. Nevertheless, functional studies are frequently difficult to perform *in vivo* with developmental programs with an extensive self-regulating GRN.

To date, most published assays of Hh activity have depended on reporter-fusions (e.g. 8X-GLIBS-luciferase) in readily transfectable cells or similar GLI-responsive markers (Myers et al., 2013). While these methods are readily quantifiable, they greatly oversimplify the known tissue interactions of developmental processes that operate*in vivo* . These cell-based assays often require the absence of Ptch and an intact GRN present in the living animal. A promising advance is the recent development of rapid assays of *SMO* activity that can determine biological activities in minutes or hours (Myers et al., 2017).

In all of our over-expression assays, we empirically determined that additional interventions were required to obtain interpretable data. Furthermore, the activation, or suppression, of the GRN obscured any differences between the variants themselves, or with the WT control. Indeed, the marker and morphological criteria we used as a biological response required many hours or days to become measurable. We observed that many of these over-expression measures were highly time-dependent (data not shown). By contrast, the ability to generate CRISPER/Cas9 mutant test lines has proven to be an immensely powerful tool. It avoids the requirement for co-interventions that can sometimes obscure the results.

Finally, recent advances have independently supported our identification human p.V404 as an important residue in the 7TM domain of *SMO* . While formally no longer considered novel (PM2), we observe that the single whole genome detection of p.V404M occurred in a distinct ethnic group for our proband. The analogous murine residue p.V408 is implicated in cholesterol agonist binding (PM1). An engineered variant p.V408F in this site sterically blocks cholesterol and abrogates GLI-luciferase reporter activity (Deshpande et al., 2019). V404, the binding pocket residues for p.V408, was reported the mutation have the potential function as alter sterol affinity or directly stabilize the active SMO conformation (Sharpe et al., 2015). While

our human variant p.V404M lacks the same bulky Phe side chain, the structural similarities are consistent with the requirements of this residue being essential in*SMO* function.

In conclusion, the value of Targeted Sequencing of the Hh pathway has established a framework for integrated genetics interpretations of the potential for gene:gene interactions that can serve as a basis for more comprehensive and accurate genetic counseling in HPE. Strict adherence to existing guidelines indicate that the driver mutations described in this report, such as *FGF8* and *SHH* , rarely require co-morbid modifiers. As whole exome and whole genome data become more readily available for HPE, we anticipate that this autosomal dominant with modifier model will continue to be subject to experimental validation and result in further insight.

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CONFLICT OF INTEREST STATEMENT.

None declared.

DATA ACCESS

All raw and processed sequence data has been submitted to the NCBI Database of Genotype and Phenotype (dbGaP), is available to IRB approved researchers studying brain development, under session number phs001653.v1.p1.

AUTHOR CONTRIBUTIONS

E.R., P.H., and M.M. have prospectively collected HPE clinical DNA samples and data. M.T., and S.H. prepared the synthetic mRNA and*in situ* probes. M.T. collected embryos, performed *in vivo*experiments and biological analysis. B.C. and R.S., from the NHGRI Zebrafish Core, designed the CRISPR/cas9 K/O model. B.C. performed experiments to create the CRISPR/cas9 K/O model. M.T., and B.C., performed genotyping. E.R., S.H., and M.T., contributed to the design of the study and data interpretation. M.T. prepared the figures. M.T., E.R., S.H., P.H. and B.C. wrote the manuscript. All authors reviewed and approved the final draft of the manuscript.

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Tables

Table 1.

Clinical and molecular findings of genetic variants in the human SMO gene.

A summary of the relevant findings for each of the six HPE probands listed by their unique identifiers. Potential co-morbid driver mutations are listed where relevant (Richards et al., 2015). Similarly, co-variants are described in detail for each *SMO* variant positive proband in Suppl.TableS8-13 using the annotation scheme published in (Roessler et al., 2018a).

Figure Legends

Figure 1.

(*A, B*) Schematic representation of the human *SMO* coding region (cDNA) and its domains: CRD, Cysteine-rich domain (green); LD, Linker domain (orange); TMD, heptahelical transmembrane domain (blue); ICD, Intracellular domain (white in A, red in B). Asterisks indicate the position for each variant in the structure of *SMO* . (*C*) Amino acid sequence alignment of *Danio rerio* , *Homo sapiens*and *Mus musculus* Smo orthologs. The residues conserved in all three species are shown in blue. The aminoacid position of each variant is shown on the top. The residues highlighted in yellow indicate the mutation site of each variant in the context of adjacent residues.

Figure 2.

Characterization of the over-expression microinjection consequences of the seven variants as assessed by *in situ* hybridization. A 2pg dose of *SHH* mRNA co-injected with 200pg of each distinct*SMO* mRNA into one-cell stage embryos. (*A*) Representative lateral view of the craniofacial region phenotype at 48hpf; cyclopia at 24hpf as an uncommon severe phenotype is shown in the upper right panel. Arrows indicate less pigmentation of the retina as a consequence of co-injection. (*B*) The biological response by over-expression analysis was measured by visual scoring of their phenotypes at 24hpf. An abnormal phenotype was observed for all variants including WT*SMO* . There is no statistically significant difference between each variant mRNA with respect to the percentage of embryos with an abnormal phenotype. (*C, D*) *In situ* hybridization of embryos with *pax6a* as eye developmental makers at 19hpf and ventral view of the expression pattern. Suppression of *pax6a*expression in the eye region is observed (C_typeII) compared with*gfp* injection (C_typeI) and quantified (D). (*E* ,*F*) *In situ* hybridization of embryos with *neurod4* as a forebrain and neural developmental maker at 24hpf. The dotted line indicates the edge of the eye and lens area. Mild reduction of*neurod4* expression in eye region is observed (E_type II) compared with *gfp* injection (E_typeI). (*G* and *H*)*In situ* hybridization of embryos with *pax2a* and ventral view of expression pattern. Enhancement of *pax2a* expression in the eye region is observed (G_typeII) compared with *gfp*injection (G_typeI) and quantified (F). Error bars represent means with SD. **P $<$ 0.01 (Student's t-test).

Figure 3.

Characterization of the loss-of-function approach of the seven variants using K/O model rescue assay. (*A*) Schematic representation of human *SMO* coding region and components of its domains. (*B*) Lateral view of the control, *smo-/smo-* and injection phenotypes observed at 48h post-fertilization. The arrowheads indicate the shortened body trunk and curled *U* -type tail phenotype, asterisk and arrow show heart edema and less eye color of retina in the small eye of the K/O phenotype. Three different rescue phenotypes of injection with *SMO* construct-derived mRNA. Type I represents a normal phenotype as seen with control and a completely rescued embryo. Type II represents partially rescued color and size of eye but not the somite. Type III is a partially rescued color and size of eye and body shape. (*C*) Results of rescue analysis. Genotyped embryos after confirming the phenotype showed that p.V404M was significantly impaired in the *smo-/smo-* phenotypic rescue compared to other variants. (*D*) The division of rescue type after genotype phenotype analysis showed the percentage of complete rescue is different only in p.V404M. Error bars represent means with SD. **P $<$ 0.01 (Student's t-test).

Figure 4.

Functional analysis of representative domain variants were conducted using low dose MO of *ptch1* and *ptch2* . WT zebrafish embryos were injected with low dose *ptch1* and *ptch2* MO as well as with each of the four distinct *SMO* mRNA transcripts and harvested at 24hpf. (*A*) The eye area was measured at 24hpf. Significant differences in eye area observed in *SMO* p.V404M when compared to*gfp* , *SMO* p.R113Q, p.R199W and p.P687L. Representative eye images of *gfp* (a) and *SMO* p.V404M (b) are shown upper panels. (*B*) Somite angle was measured at 24hpf. Significant difference in somite angle observed in *SMO* p.R113Q when compared to *gfp* , *SMO* p.R199W and p.P687L. Representative somite images of *gfp* (a') and *SMO* p.R113Q (b') are shown upper panels. Error bars represent means with SD. *P $<$ 0.05 (Student's t-test).

Suppl. TableS1

Number of embryos scored in the pilot study for our overexpression experiment.

Suppl. TableS2

Number of embryos with each phenotype in Figure.2B.

Suppl. TableS3

Number of embryos with each phenotype or expression pattern in Figure.2B_Cyclopia, D,F and H.

Suppl. TableS4

Number of embryos in our rescue experiment in Figure 3.

Suppl. TableS5

Number of embryos scored in pilot study for K/D experiment using low dose *ptch1 or 2* MO respectively.

Suppl. TableS6

Number of embryos scored in pilot study for K/D experiment using a combination injection.

Suppl. TableS7

The driver mutation and co-morbid inherited coding variants (benign, variant of unknown significance [VOUS], or pathogenic) and non-coding variants conserved among at least 4 of 7 vertebrates (ultra-conserved). Co-occurring damaging coding findings (see below) are listed as potential modifier variants using the longest transcript for uniform numbering. For effects on overlapping transcripts see Suppl. TablesS8-13, column AL. Justifications for assertions of coding region pathogenicity incorporate the principal accepted guidelines in (Richards et al., 2015) for a simple autosomal dominant disorder with high penetrance. Variants with an allele frequency greater than 1:10,000 (the live birth incidence of HPE in the newborn nursery) were annotated by an asterix (*) interpreted as likely of minor effect. Potential modifiers were not filtered by allele frequency. However, variants with a Minor Allele Frequency >1% were omitted from the analysis. Similarly, non-coding co-morbid variants scored as >4 (see below) are also annotated as potential modifier loci.

Suppl. TableS8-13

Genotype findings in each of the six probands are annotated and analyzed for common measures of pathogenicity and inheritance (Roessler et al., 2018a). Coding variants were annotated using dbNSFP v.3.3a consensus (http://annovar.openbioinformatics.org/en/latest/): determined by consensus >50% of [SIFT, PolyPhen2HDIV, PolyPhenHVAR, LRT, MutationTaster, MutationAssessor, FATHMM, PROVEAN, FATHMM-MKL, MetaSVM, MetaLR; see columns CC to CP and CV to CZ] as driver mutations (red), damaging variants (pink) or <50% as likely benign (green). Pathogenic mutations (red) cluster at discrete HPE loci. Variants observed at a Minor Allele Frequency (MAF) >1% as determined by ExAC [http://exac.broadinstitute.org] were removed from the dataset and assumed to be benign. Non-coding SNPs were prioritized based on a measure of phylogenetic conservation (our scale of 0 to 7, with 7 being the highest rank considered) among seven vertebrate species [danRer7, fr3, TetNig1, galGal3, mm10, morDom5, xenTro3] in the current version of the ECR browser [ecrbrowser.dcode.org]. Non-coding variants with conservation <4/7 (light blue) are by far the majority among SNPs as well as novel observations. Ultra-conserved variants (>4/7, dark blue) represent a minority of findings, but interestingly are common in the vicinity of known HPE genes. Indels [colored in tan] were scored [0 to 7] based on the coordinate score immediately 5' of the indel variation. Most non-coding variation is poorly conserved [light blue, score 0 to 3). Ultra-conserved variants [dark blue, score >4] are far less common, but can be seen within or near a known HPE gene. Our score [0 to 7] was then compared with other position specific metrics [gerp++, phyloP100, phyloP46, CADDRawscore, CADD13, CADDindel, dann, FATHAMM_coding, FATHMM_noncoding, and Eigen; see columns BM to BW]. The only filters currently used are [Func.ref: no ncRNAexonic, no ncRNAintronic; and Exonicfunc.refGene: the synonymous coding region changes; see columns AH and AK]. Known repeat regions determined by the ECRbrowser analysis were removed from the dataset. Poor data was colored in grey but retained in the dataset.

Suppl. Figure 1.

The Hh GRN. Note the negative feed-back loop between *SMO*signaling and *PTCH* 1/2. While low dose *SHH* would be expected to lower *PTCH1/2* on the surface of responding cells, the knock-down of *PTCH1/2* via MO follows a distinct time course. In*ptch* K/D experiments the optimal *SMO* dose is reduced by over half compared with low dose *SHH/SMO* co-injection. Hence, the changes in morphology and gene expression are not necessarily the same.

Suppl. Figure 2

The results of *SMO* mRNA dose-response pilot experiments for our over-expression bioassay development. Abnormal phenotypes are not consistently observed with single injections of low dose of *SHH*or even a high dose of *SMO* . On the other hand, a 2pg of*SHH* and 200pg of *SMO* mRNA (co-injection) significantly induced abnormal phenotypes compared with all other dose conditions [except 1pg of *SHH* and 600pg of *SMO*]. For this pilot study, the injections for 50 embryos on each group were performed in triplicate and on different days. Error bars represent means with SD. $^{**}P < 0.01$ (Student's t-test).