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# SMCHD1 mutations associated with a rare muscular dystrophy can also cause isolated arhinia and Bosma arhinia microphthalmia syndrome

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1 **Mutations in *SMCHD1* are Associated with Isolated Arhinia, Bosma Arhinia**  
2 **Microphthalmia Syndrome, and Facioscapulohumeral Muscular Dystrophy Type 2**

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**ABSTRACT**

Arhinia, or absence of the nose, is a rare malformation of unknown etiology that is often accompanied by ocular and reproductive defects. Sequencing of 38 arhinia subjects from 36 independent families revealed that 86% of independent subjects harbor a missense mutation in a constrained ATPase domain of *SMCHD1*. Mutations in *SMCHD1* also cause facioscapulohumeral muscular dystrophy type 2 (FSHD2) via a complex *trans*-acting loss-of-function epigenetic mechanism. Arhinia subjects had comparable DNA hypomethylation patterning to FSHD2 subjects, and CRISPR/Cas9 editing of *smchd1* in zebrafish yielded arhinia-relevant phenotypes. Mutations in *SMCHD1* thus contribute to remarkably distinct phenotypic spectra from craniofacial and reproductive disorders to muscular dystrophy, which we speculate to be consistent with oligogenic mechanisms resulting in pleiotropic outcomes.

123 Arhinia, or the complete absence of the external nose, is a rare congenital malformation with  
124 only 80 patients without holoprosencephaly reported in the past century (see **Supplementary**  
125 **Table 1** for all previous reports). This severe craniofacial dysmorphism can be isolated or  
126 accompanied by other craniofacial defects including coloboma, anophthalmia, cataracts,  
127 nasolacrimal duct atresia, choanal atresia, and cleft palate (**Fig. 1**). Seventeen patients with  
128 arhinia and ocular defects have been reported with coexistent reproductive failure secondary to  
129 hypogonadotropic hypogonadism, a triad called Bosma arhinia microphthalmia syndrome  
130 (BAM; OMIM 603457)<sup>1</sup>. In the neonatal period, patients with arhinia are at high risk for  
131 respiratory distress, difficulty feeding, and sepsis (as a complication of reconstructive surgery),  
132 but those surviving infancy generally demonstrate normal cognitive development with few  
133 functional challenges limited to vision loss, the stigma of facial deformities, and osteoporosis  
134 and infertility due to hypogonadism. The rarity of these malformations and cross-disciplinary  
135 nature of its comorbid conditions have limited systematic efforts to catalog its associated  
136 phenotypes, although these comorbidities suggest that genetic factors influencing this condition  
137 may have broader developmental implications.

138

139 Genetic studies of arhinia have been limited to karyotype analysis, chromosomal microarray, and  
140 candidate gene approaches targeting genes related to neural crest cells (NCC) or craniofacial  
141 placodal development; to date, no causal locus has been identified. Phenocopies are likewise  
142 scarce; homozygous null mutations in *Pax6* arrest nasal placodogenesis in mice<sup>2</sup> and cause  
143 rudimentary or malformed noses in humans<sup>3-5</sup>. However, heterozygous and homozygous null  
144 mutations in *PAX6* cause aniridia and severe structural brain abnormalities, respectively, that are  
145 not observed in individuals with arhinia<sup>3-5</sup>. We formed an international consortium to investigate

146 the genetic etiology of arhinia and its associated comorbidities and aggregated all available cases  
147 across sites. We sequenced 38 individuals with arhinia from 36 independent families as well as  
148 51 family members without arhinia. Through family-based analyses of *de novo* mutations and  
149 genome-wide burden analysis supported by functional studies, we report that rare missense  
150 variants in *SMCHD1* represent the predominant single gene contributor to arhinia. Notably,  
151 *SMCHD1*, an epigenetic repressor, has also been implicated in a rare, complex oligogenic form  
152 of muscular dystrophy (fascioscapulohumeral muscular dystrophy; FSHD2, OMIM 158901).  
153 Methylation studies in arhinia patient samples, as well as complementation testing of arhinia  
154 variants in a zebrafish model, revealed a common direction of allele effect in both arhinia and  
155 FSHD2, a surprising observation considering the striking difference in phenotypes. Given the  
156 known oligogenic architecture of FSHD2, these data argue that loss-of-function at the *SMCHD1*  
157 locus contributes to the diverse manifestations of arhinia, BAM, and FSHD2, likely through  
158 interaction with other genomic loci.

159

## 160 **RESULTS**

### 161 **Samples, phenotypes, and epidemiology of arhinia**

162 We established a large international consortium and aggregated all available biospecimens and  
163 clinical data to identify the genetic cause of arhinia. This cohort encompassed 24% of all 80  
164 previously reported subjects and an additional 19 new subjects (**Supplementary Table 1**),  
165 facilitating a relatively comprehensive picture of the phenotypic spectrum of arhinia  
166 (**Supplementary Table 2**). All subjects had complete arhinia, almost universally accompanied  
167 by abnormalities of the surrounding craniofacial structures, including high-arched or cleft palate,  
168 absent paranasal sinuses, hypoplastic maxilla, nasolacrimal duct stenosis or atresia, and choanal

169 atresia (**Fig. 1**), and 44% of subjects also had dysmorphic pinnae or low-set ears. Ocular  
170 phenotypes included anophthalmia or microphthalmia (69%), uveal coloboma (76%), and  
171 cataract (47%), while at least six subjects had normal eye anatomy and vision. Among the 28  
172 subjects in whom the reproductive axis could be assessed (19 male; 9 female), all demonstrated  
173 reproductive failure due to hypogonadotropic hypogonadism (HH), and all seven subjects with  
174 available brain MRI data presented with absent olfactory structures on imaging; both  
175 presentations are hallmark clinical signs of gonadotropin releasing hormone (GnRH) deficiency  
176 and anosmia (Kallmann syndrome; OMIM 308700). Twenty-four of these 28 individuals also  
177 had ocular defects, indicating that 86% of arhinia subjects that could be assessed met diagnostic  
178 criteria for BAM.

179

### 180 **Sequencing and gene discovery**

181 To investigate the contribution of rare coding variants to arhinia, we used whole-exome  
182 sequencing (WES) in an initial cohort of 22 probands: 9 subjects with available DNA from  
183 families of varying pedigree configurations and 13 subjects with no familial samples available  
184 (see **Supplementary Fig. 1**). Concurrent with WES, whole-genome sequencing (WGS) was  
185 performed in four members of a previously reported multiplex family that included a proband  
186 affected with arhinia, an affected sister, an unaffected brother and father, a mother with anosmia  
187 and subtle nasal and dental anomalies, a maternal half-aunt with arhinia, and a maternal  
188 grandmother who also had mild nasal and dental anomalies (**Supplementary Fig. 1**; family  
189 O)<sup>6,7</sup>. These analyses identified rare missense variants in *SMCDH1* in 81.8% of independent  
190 probands (**Table 1**), none of which were present in the Exome Aggregation Consortium (ExAC)  
191 database of 60,706 healthy individuals with WES<sup>8,9</sup>. Among the eight WES samples with

192 familial information and an observed *SMCHD1* mutation, we confirmed 3 to have arisen *de novo*  
193 and additional 4 samples where the mutation was not observed in available family samples  
194 (parent or siblings), while in one subject (family T), the variant was inherited from a father with  
195 no craniofacial abnormalities but who carried a clinical diagnosis of muscular dystrophy. To  
196 formally test whether the observed allelic distribution in arhinia subjects represented a significant  
197 accumulation of rare missense variants, we compared the rare mutation burden among 22,445  
198 genes in the arhinia subjects to the WES data from ExAC (minor allele frequency [MAF]  
199 <0.1%). Powered by the size of our aggregate cohort, we found that *SMCHD1* was the only gene  
200 that achieved genome-wide significance for a rare mutation burden ( $p = 2.9 \times 10^{-17}$ , **Fig. 2**).

201  
202 All of the variants identified in this initial cohort were localized to six of the 48 exons that  
203 comprise *SMCHD1* (exons 3, 8, 9, 10, 12, 13; Ensemble transcript ENST00000320876). Based  
204 on this narrow distribution, we performed targeted sequencing of these exons in an additional 13  
205 subjects and discovered rare *SMCHD1* missense mutations in 9 of these probands. WES on the  
206 *SMCHD1* negative samples identified additional rare missense mutations in three of the four  
207 probands remaining, all localized to exons adjacent to the initial six screened in the targeted  
208 assays (exons 5,6,11). In these collective analyses (WES, WGS, targeted sequencing), 86.1%  
209 (31/36) of independent arhinia probands had a rare missense variant in *SMCHD1* (**Table 1**), and  
210 all sporadic subjects with complete trios harbored a *de novo* variant ( $n = 10$ ). In an additional  
211 four multigenerational, multiplex families (O, T, AB, AH) harbored rare missense alleles in  
212 *SMCHD1* that segregated with variable phenotypes such as anosmia, asymmetric nares,  
213 abnormal dentition, nasal hypoplasia, hypogonadism, and muscular dystrophy, suggesting



214 incomplete penetrance, variable expressivity, and possible pleiotropy associated with alterations  
215 of *SMCHDI* (**Supplementary Fig. 1a**).

216

217 *SMCHDI* is among the most highly constrained genes in the genome, suggesting strong  
218 intolerance to loss-of-function variation (evolutionary constraint pLI = 1.00)<sup>10</sup>, with an estimated  
219 combined prevalence of 1 in 10,000 heterozygous null individuals in ExAC. However, the gene  
220 does not show particularly strong intolerance to missense variation (81% of expected missense  
221 variants observed; p = 0.016). The localization of all arhinia-specific *SMCHDI* mutations to  
222 exons 3-13 led us to probe further the distribution of rare missense variants observed in ExAC  
223 across this gene. Analyses of regional constraint among the individual exons and critical domains  
224 in the protein revealed strong evidence of constraint against missense variation in the 5' region  
225 of the gene, including exons 1-19 encompassing an ATPase domain (61% of expected missense  
226 variants observed;  $\chi^2 = 32.40$ ; p =  $1.26 \times 10^{-8}$ ), whereas there was no evidence of in the region  
227 including exons 20-48, encompassing an SMC-hinge domain (95% of expected missense  
228 variants observed;  $\chi^2 = 0.86$ ; p = 0.36; **Fig. 3**). This observation of strong regional constraint is  
229 consistent with the increased burden of rare *SMCHDI* alleles in arhinia subjects, and suggests  
230 that these alleles may impede protein function. *In silico* prediction of protein pathogenicity from  
231 the Combined Annotation Dependent Depletion (CADD) database revealed that the 19 arhinia-  
232 specific *SMCHDI* variants were more deleterious than all rare, nonsynonymous variants in  
233 ExAC (MAF < 0.01%, ExAC n = 378, p =  $8.27 \times 10^{-5}$ ; Supp Figure 2). Importantly, there are 20  
234 rare missense variants in ExAC between exons 3-13 with CADD scores exceeding the median  
235 arhinia score (16.91), further supporting our speculation that deleterious *SMCHDI* variant are  
236 not fully penetrant, and such variants alone may not be sufficient to manifest arhinia.

237 **Mutational overlap between arhinia and a rare form of muscular dystrophy**

238 *SMCHD1* encodes a large protein (2007 amino acids) containing a 5' functional GHKL-type  
239 ATPase domain<sup>11</sup> and a 3' SMC-hinge domain (for dynamic DNA binding) that serves as an  
240 epigenetic regulator of both autosomal and X-linked genes<sup>12-15</sup>. The discovery of an association  
241 between this gene and craniofacial development was unexpected since mutations in *SMCHD1*  
242 are associated with FSHD2, a rare oligogenic form of muscular dystrophy. In FSHD2,  
243 heterozygous loss of *SMCHD1* repressor activity, in combination with a permissive D4Z4  
244 haplotype on chromosome 4 (4q35), allows for the ectopic expression of the DUX4 protein  
245 which is cytotoxic to skeletal muscle<sup>16</sup>. The distribution of mutations in FSHD2 span the entire  
246 gene and include missense and truncating variants, whereas all variants observed in arhinia  
247 subjects were missense variants clustered tightly around the GHKL-type ATPase domain (**Fig.**  
248 **3**), which is thought to be critical to the controlled release of DNA bound by SMCHD1<sup>17</sup>.  
249 However, we were surprised to find several previously reported FSHD2-specific missense  
250 mutations localized to exons 3-13, and one of these FSHD2 variants was also detected in the  
251 arhinia cohort (G137E in subject #AG1)<sup>18</sup>. At present, neither subject has features of both  
252 disorders, indicating that either these phenotypes have arisen by divergent mechanisms or are  
253 influenced by additional loci.

254

255 **Methylation profiling and protein expression in arhinia and FSHD2 subjects with**  
256 ***SMCHD1* variants**

257 Haploinsufficiency and dominant negative loss-of-function models have both been invoked in  
258 FSHD2 for *SMCHD1* mutations that disrupt the open reading frame (nonsense, indel, or splice-  
259 site) or preserve it (missense), respectively<sup>18</sup>. In both models, loss of SMCHD1 repressive

260 activity manifests as a decrease in DNA methylation at SMCHD1 binding sites<sup>11,16,19,20</sup>. Clinical  
261 testing for FSHD2 relies on methylation profiling of two of these binding sites, the FSHD-  
262 associated 4q35 D4Z4 macrosatellite array and the highly homologous 10q26 D4Z4 array<sup>21,22</sup>.  
263 To pursue evidence of mechanistic overlap between arhinia and FSHD2, we quantified 4q35  
264 D4Z4 methylation in 23 arhinia subjects (19 with *SMCHD1* rare missense variants) and 22  
265 family members: 4 with *SMCHD1* rare missense variants, including two with anosmia, one with  
266 a hypoplastic nose, and one with symptoms of muscular dystrophy, while the remaining 19  
267 family members were *SMCHD1* mutation-negative. Remarkably, 73.6% (14 of 19) of arhinia  
268 subjects with an *SMCHD1* variant had D4Z4 hypomethylation characteristic of FSHD2 (**Fig. 4;**  
269 **Supplementary Table 4**), while all 4 arhinia subjects without a rare missense variant in  
270 *SMCHD1* had normal methylation patterns. Two of the four family members harboring an  
271 *SMCHD1* variant also displayed D4Z4 hypomethylation, while 17 of the 19 family members  
272 without a rare *SMCHD1* variant had normal methylation patterns. These data confirmed that  
273 arhinia-specific mutations in *SMCHD1* were associated with the same methylation patterning at  
274 D4Z4 as seen in FSHD2, illuminating that two completely distinct phenotypes can arise from  
275 alterations to the same genetic locus, and indeed the same alleles, proposing that similar loss-of-  
276 function genetic mechanisms may result in distinct phenotypes. We thus turned to *in vivo*  
277 modeling to probe the functional impact of *SMCHD1* alterations in animal models.

278

### 279 ***In vivo* modeling studies of *SMCHD1* alterations**

280 To test directly the effect of missense alleles in arhinia patients, and to provide biological  
281 evidence for their pathogenicity, we evaluated phenotypes relevant to isolated arhinia and BAM  
282 in zebrafish (*Danio rerio*) larvae. While there is no zebrafish structure credibly homologous to

283 the human nose, facial cartilage patterning is one possible proxy for human craniofacial  
284 architecture<sup>23</sup>. Zebrafish eye development is also highly conserved between species, making the  
285 zebrafish a robust model for the study of microphthalmia gene candidates identified in human  
286 studies<sup>24-26</sup>. *D. rerio* further possesses at least two of the three GnRH paralogs that exist in  
287 humans, and the processes by which neurons proliferate, migrate, and maintain the  
288 neuroendocrine axis are thought to be largely conserved between humans and teleosts<sup>27-29</sup>.  
289 Importantly, the zebrafish genome harbors a single SMCHD1 ortholog (49% identical, 67%  
290 similar to human), and the N-terminal encoding the ATPase domain is conserved between the  
291 two species (**Supplementary Fig. 2**).

292

293 We designed two non-overlapping morpholino (MO) antisense oligonucleotides targeting splice  
294 donor sites of two different exons within the *smchd1* genomic region encoding the ATPase  
295 domain (e3i3 and e5i5 targeting exons 3 and 5, respectively). The e3i3 or e5i5 *smchd1* MOs  
296 (3ng, 6ng, and/or 9 ng/embryo) were injected into embryo batches at the one-to-two cell stage  
297 and larvae were evaluated quantitatively for aberrant cartilage patterning, ocular development,  
298 and reproductive axis integrity between 1.5 and 3 days post-fertilization (dpf) (**Fig. 5a**). All  
299 morphant batches demonstrated a dose-dependent decrease in ethmoid plate width (**Fig. 5a, 5b**,  
300 **Supplementary Fig. 3a**); a dose-dependent increase in ceratohyal arch angle and delayed (or  
301 absent) development of ceratobranchial arch pairs (**Fig. 5a, 5c, 5d, Supplementary Fig. 3b-c**)  
302 and microphthalmia (tested at a 9 ng dose), all of which demonstrate alterations to phenotypes of  
303 relevance to human craniofacial and ear development. Moreover, whole-mount immunostaining  
304 of MO-injected embryos with a pan-GnRH antibody followed by ventral imaging revealed a  
305 prominent phenotype. The morphant olfactory bulbs and hypothalami were intact; however, the

306 average projection length of the terminal nerve, where GnRH3 neurons reside, was reduced by  
307 45% compared with controls ( $p < 0.0001$ ;  $n = 20$  embryos/batch; 2 measurements/embryo,  
308 repeated with masked scoring; **Fig. 5g**). The observed cartilage, eye, and GnRH phenotypes  
309 were unlikely to be non-specific as each defect was reproduced with both MOs tested, but co-  
310 injection of MO and full-length human wild-type (WT) *SMCHD1* mRNA rescued each  
311 phenotype significantly (**Fig. 5**). To further confirm these findings and rule out artifacts of MO  
312 suppression or toxicity, we targeted the *smchd1* locus using CRISPR/Cas9 genome-editing to  
313 generate small insertions and deletions of the coding sequence (**Supplementary Fig. 4**); each of  
314 the craniofacial, ocular, and GnRH defects observed in the two morphant models were  
315 significantly recapitulated in F0 mutants (Figure 5, **Supplementary Fig. 5**).

316

317 Having established credible quantitative *in vivo* assays, we tested both gain and loss of function  
318 paradigms. Injection of either full-length human WT SMCHD1 mRNA or equivalent doses of  
319 full-length human mRNA bearing three different recurrent arhinia-associated variants (S135C,  
320 L141F, and H348R) into zebrafish embryos independent of MO did not yield appreciable  
321 craniofacial phenotypes (**Supplementary Fig. 6**). Moreover, augmented doses of mutant mRNA  
322 alone (up to 100 pg) or combinatorial injections of mutant and WT mRNA (100 pg each) was  
323 likewise unremarkable arguing that, at least in the context of this assay, a gain-of-function  
324 biochemical mechanism is unlikely. Given that suppression of *smchd1* reproduced the three  
325 hallmark phenotypes of BAM, we next tested a loss of function paradigm through *in vivo*  
326 complementation. Focusing on our most sensitive assay, the quantitatively defined reduced  
327 projection of the GnRH-positive terminal nerve, we co-injected either: (1) full-length human WT  
328 *SMCHD1* mRNA; (2) human message encoding each of the three variants identified recurrently

329 in arhinia subjects (S135C, L141F, or H348R); or (3) human message encoding a missense  
330 variant (P690S) that causes FSHD2<sup>16</sup> with the e5i5 MO. Full-length human WT *SMCHD1*  
331 mRNA, but none of the mutant mRNAs associated with arhinia or FSHD2, rescued the GnRH  
332 phenotype (**Fig. 5g**). Complementation of message with a common, and presumably benign,  
333 variant from ExAC (V708I; rs2270692) also rescued the phenotype, supporting assay specificity.  
334 The likely mode of action of the discovered arhinia alleles in this assay is thus loss-of-function,  
335 and we find no foundational differences between the arhinia-specific alleles and the alleles  
336 discovered in FSHD2 patients with respect to direction of effect.

337

338 Next, we performed CRISPR/Cas9 genome editing on mouse embryos using a guide RNA  
339 spanning the boundary between exon 3 and intron 3 of mouse *Smchd1*. The 63 embryos  
340 recovered after two zygotic injection sessions displayed a range of variants including WT,  
341 homozygous knock-ins of L141F (KI), homozygous knock-outs (KO), compound heterozygotes  
342 (L141F/null), and complex compound heterozygous deletions (**Supplementary Table 3**).  
343 Unfortunately, multiple attempts to generate non-mosaic L141F heterozygous embryos (WT/KI),  
344 akin to what is observed in arhinia subjects, using WT repair templates at equimolar ratio to the  
345 mutant repair templates were unsuccessful. Non-mosaic homozygous KO, homozygous KI, and  
346 compound heterozygous KO/KI embryos were examined using optical projection tomography<sup>30</sup>  
347 at 13.5 days post-conception (dpc) and we observed no morphological or growth anomalies  
348 (**Supplementary Fig. 5**). These results in mouse embryos do not support a simple  
349 haploinsufficiency, or indeed null, mechanism causing arhinia but are consistent with previous  
350 studies in mice in which *Smchd1* knockdown did not cause craniofacial defects<sup>12,31</sup>, though  
351 complete knockout of the gene is not viable, and support the notion from the human genetic data

352 that alteration to a single copy of *SMCHD1* alone may not be sufficient to induce pathology in  
353 mammals.

354

### 355 **Protein modeling and human expression studies**

356 We investigated the potential impact of these arhinia-specific mutations on SMCHD1 protein  
357 structure. The protein structure of the N-terminal region of SMCHD1, where the constrained  
358 GHKL-type ATPase domain resides, is unknown. However, the crystal structure of heat shock  
359 protein 90 (Hsp90), a member of the GHKL-ATPase protein family found in yeast, is known  
360 (PDB: 2CG9), and a recent small-angle X-ray scattering study demonstrated that the ATPase  
361 domains of these two proteins are similar in structure<sup>11</sup>. We generated a structural model of the  
362 N-terminal region of SMCHD1 with Phyre2<sup>32</sup> (**Fig. 6A**), with residues mutated in arhinia and  
363 FSHD2 highlighted. The top ranking templates identified were Hsp90 structures, covering  
364 residues 115-573, although the strongest homology is from approximately residues 120-260. The  
365 structural model indicates that the arhinia-specific mutations tend to cluster on the protein  
366 surface, suggesting that residues mutated in arhinia may be part of an interaction surface. This  
367 hypothesis is independently supported by sequence-based predictors of solvent accessibility (**Fig.**  
368 **6B**), which reveal a significant tendency for arhinia mutations to be exposed on the protein  
369 surface.

370

371 Finally, as an initial step towards understanding the pathogenic mechanism of arhinia, we  
372 measured SMCHD1 protein levels and performed RNAseq on lymphoblastoid cell lines (LCLs)  
373 from 23 total subjects: 10 subjects with arhinia harboring presumably pathogenic *SMCHD1*  
374 variants, 11 unaffected family members without *SMCHD1* mutations, and two family members

375 with a mutation in *SMCHD1* and anosmia or a hypoplastic nose (AH3 and AH5, respectively). In  
376 the arhinia subjects, *SMCHD1* protein levels appeared to be preserved (on average) in LCLs  
377 from subjects with an *SMCHD1* variant compared to controls using two different anti-Smchd1  
378 antibodies (Bethyl A302-872A-M and Abcam ab122555; **Supplementary Fig. 9**). RNAseq  
379 analyses incorporated affection status and familial relationships, and differential expression  
380 analyses following permutation testing revealed a relatively uniform distribution of p-values  
381 compared to expectations. We first compared overall expression changes in *SMCHD1* and allele-  
382 specific expression differences of *SMCHD1* transcripts in arhinia probands. After confirming all  
383 mutations in the expressed transcripts that were observed in the DNA analyses, we found that  
384 arhinia subjects demonstrated a slight, non-significant decrease in *SMCHD1* mRNA expression  
385 compared to controls (fold-change = 0.94,  $p = 0.49$ ), with no average difference in allelic  
386 expression of the missense variant compared to the reference allele ( $p = 0.70$ ), indicating no  
387 change in message stability in arhinia subjects, at least in the available biomaterials (LCLs).

388

389 We next looked for pathways and networks that may be associated with manifestation of arhinia.  
390 When considering all differentially expressed genes following permutation testing (unadjusted  $p$   
391  $< 0.05$ ; **Supplementary Table 5**), we discovered alterations to multiple pathways and human  
392 phenotypes associated with craniofacial development and epigenetic modification. Remarkably,  
393 the strongest human phenotype associated with these genes from ToppGene pathway analysis<sup>33</sup>  
394 was “depressed nasal tip” ( $p = 5.1 \times 10^{-5}$ ), as well as related phenotypes such as absent nasal  
395 septal cartilage, semilobar holoprosencephaly, small placenta, and median cleft lip and palate ( $p$   
396  $< 0.005$ ). Encouraged by these initial network results across all genes, we next sought greater  
397 specificity of these networks by integrating orthogonal chip and RNAseq data generated from



398 *SMCHD1*-null mouse neural stem cells (NSCs)<sup>13</sup>. From these analyses, we observed a significant  
399 enrichment of down-regulated genes between the human and mouse datasets ( $p = 0.029$ ), but not  
400 up-regulated genes ( $p = 0.40$ ), and we identified a high-confidence set of nine overlapping genes  
401 that were down-regulated in both datasets; the same phenotype, “depressed nasal tip”, was more  
402 significant than in the human data alone ( $p = 2.1 \times 10^{-5}$ ). We found these results, and multiple  
403 related human phenotype associations, to be primarily driven by two genes (*TGIF1*,*DOK7*), both  
404 of which have already been demonstrated to play a role in craniofacial morphogenesis. *DOK7*  
405 haploinsufficiency causes fetal akinesia deformation sequence (FADS; OMIM 208150). The  
406 phenotypic spectrum of this disorder includes many features of BAM (depressed nasal bridge,  
407 cleft palate, choanal atresia, microphthalmia, cataract, coloboma, cryptorchidism, and absent  
408 olfactory structures)<sup>34</sup>, and heterozygous loss-of-function mutations in *TGIF1* cause  
409 holoprosencephaly-4 (OMIM 142946), which may include arhinia, microphthalmia, and cleft  
410 palate<sup>35</sup>. Notably, two additional genes associated with the “depressed nasal tip” phenotype, *ICK*  
411 and *KDM6A* (an X-linked gene previously associated with Kabuki syndrome, including multiple  
412 craniofacial anomalies and cleft palate)<sup>36,37</sup> were also differentially expressed in the human  
413 dataset. These four genes are therefore rational mechanistic candidates for modifiers of the  
414 arhinia phenotype in the presence of *SMCHD1* mutations.

415

#### 416 **Prediction of phenotypic outcomes**

417 Among the most striking findings in this study was the demonstration that variants in the same 5’  
418 constrained region of *SMCHD1* are associated with both FSHD2 and arhinia, and the discovery  
419 of an identical amino acid substitution (G137E) within this region that was associated with both  
420 phenotypes<sup>18</sup>. Methylation assays suggested indistinguishable hypomethylation signatures

421 between arhinia and FSHD2 probands. To our knowledge, the comorbid presentation of arhinia  
422 and FSHD2 has never been reported. The lack of previously reported FSHD2 symptoms in  
423 arhinia subjects may be a consequence of the oligogenic architecture of FSHD2, which would  
424 suggest that only a small subset of subjects with arhinia, an already rare condition, would harbor  
425 the requisite genetic architecture at D4Z4 and thus be both at risk and past the average age at  
426 onset for FSHD2. In addition, features such as facial weakness, which is often one of the first  
427 clinical signs of FSHD2, could easily be overlooked or dismissed in a patient with craniofacial  
428 anomalies who has undergone multiple corrective surgeries. Nonetheless, we performed analyses  
429 comparable to clinical diagnostic testing and found 2 arhinia probands (A1 and E1) with  
430 *SMCHD1* variants who met the four critical clinical criteria for susceptibility to FSHD2: 1) an  
431 *SMCHD1* pathogenic variant, 2) D4Z4 hypomethylation (bisulfite sequencing [BSS] <25%), 3) a  
432 permissive chromosome 4q haplotype, and 4) an 11-28 D4Z4 repeat unit at the 4q array  
433 (**Supplementary Table 4**)<sup>16,21,38,39</sup>. Five other subjects may be at risk for FSHD2 but will require  
434 additional confirmatory clinical testing. We had consent to re-contact both arhinia subjects  
435 meeting FSHD2 clinical criteria, and phenotypic evaluation suggested that at least one subject  
436 had symptoms of FSHD2, proposing yet another mutation site (N139H) common to these two  
437 disorders. Overall, these results suggest that at least two mutations (G137E and N139H), in the  
438 presence of a specific genetic background, can manifest as two divergent clinical phenotypes.

439

## 440 **DISCUSSION**

441 We describe genetic, genomic, and functional evidence that implicate *SMCHD1* as the  
442 predominant driver of arhinia in humans. These analyses represent the first evidence of a genetic  
443 cause for this rare craniofacial malformation. Through a large collaborative effort, we were able

444 to combine data from a sizeable fraction of subjects reported in the literature (24%) and 19 new  
445 subjects, which facilitated the uniform evaluation of the clinical phenotype associated with this  
446 condition. We find that 86% of subjects with arhinia who could be assessed present with the  
447 BAM triad, and that 88% of subjects with BAM harbored *SMCHD1* variants. In addition, the  
448 three BAM subjects without an *SMCHD1* variant were either part of a consanguineous family, or  
449 exhibited unique phenotypic features (e.g., tracheoesophageal fistula) suggesting alternative  
450 genetic causes in these individuals. Our findings thus suggest a novel role for *SMCHD1* in  
451 cranial NCC migration and/or craniofacial placodal development.

452

453 Our genetic observations raise questions concerning potential molecular mechanisms that lead  
454 mutations in the same gene to produce the distinct phenotypes. The fact that all of the arhinia-  
455 associated mutations are missense changes rather than truncating mutations, as often seen in  
456 *FSHD2*, suggests that the arhinia mechanism in humans requires production of a mutant protein  
457 rather than simple loss of function of one allele seen in *FSHD2*. However, the overlap of some  
458 arhinia mutations with missense alterations observed in *FSHD2* suggests that the mutant protein  
459 that is produced is indeed deficient in some critical function. The *FSHD2* hypomethylation  
460 signatures associated with *SMCHD1* mutations are demonstrably loss-of-function and consistent  
461 in most circumstances with a haploinsufficiency model, although dominant-negative activity of  
462 the mutant protein has been suggested as the cause of a more severe phenotype in some  
463 cases<sup>16,18,20</sup>. We find largely identical methylation patterns at the *D4Z4* repeat region on  
464 chromosome 4 in arhinia probands and *FSHD2* patients, supporting the view that loss of this  
465 function of *SMCHD1* also occurs in arhinia, and so does not in itself explain the difference in  
466 phenotypic outcome. Thus, additional factors must be involved in producing this distinction,

467 such as interaction at the genetic level with variants at other loci or a function-altering interaction  
468 at the protein level of the mutant SMCHD1 protein. Indeed, we found that the arhinia-specific  
469 variants tend to cluster on the surface of the protein, potentially facilitating disruption of  
470 interactions with protein partners, either wild-type SMCHD1 or other members of its complexes  
471 (or both). Correspondingly, we found no significant difference in average protein expression  
472 between arhinia probands and unaffected individuals, suggesting that the bioactivity of the  
473 protein is the critical factor in humans rather than the total amount of protein.

474

475 The distinct findings in two model systems reinforce the complexity suggested in humans. The  
476 zebrafish model supports the involvement of loss of function as both MO suppression and  
477 mosaic ablation of *smchd1* result in BAM-related phenotypes, the most dramatic of which is the  
478 GnRH terminal nerve projection defect. These results are specific, as these phenotypes are  
479 rescued with full-length human *SMCHD1* WT mRNA, but not mRNA containing recurrent  
480 arhinia mutations, and substantial overexpression confers no discernible phenotype. In the  
481 mouse, complete loss of function has been achieved as homozygosity for an exon 23 *Smchd1*  
482 nonsense mutation which produces hypomethylation and mid-gestational lethality in females<sup>12</sup>,  
483 although males are viable. Heterozygosity for this mutation, like induction of either deletions or  
484 arhinia-relevant point mutations by CRISPR/Cas9, produced no phenotypes in mouse.  
485 Unfortunately, we were unable to replicate in the mouse the heterozygous missense genotype  
486 characteristic of human arhinia (**Supplementary Fig. 5**). The fact that loss of *smchd1* is  
487 sufficient produce BAM-relevant phenotypes in the zebrafish, but loss of *Smchd1* in the mouse  
488 does not, reinforces the need to consider genetic and functional interactions of the mutant protein  
489 in causing the human arhinia phenotype. Notably, both the zebrafish and mouse genomes lack

490 recognizable orthologs of *DUX4*, the genetic interactor necessary for the development of  
491 FSHD2<sup>40,41</sup>.

492

493 The complex oligogenic architecture of FSHD2 suggests that only a small fraction of individuals  
494 with arhinia, which is exceedingly rare on its own, will have an *SMCHD1* mutation and also  
495 carry a permissive 4q35 haplotype, placing them at risk for FSHD2. Our analyses identified  
496 seven subjects that are potentially at risk for FSHD2, and at least one appears to display  
497 symptoms of the disorder. Nonetheless, one-quarter of individuals who meet genetic diagnostic  
498 criteria for FSHD2 are clinically asymptomatic, indicating that the full complement of genetic  
499 requirements for developing clinical FSHD2 is not yet known<sup>42</sup>. Like our data, the absence of  
500 arhinia in patients with FSHD2 with *SMCHD1* mutations within the constrained ATPase domain  
501 argues that loss of SMCHD1 activity alone is not sufficient to produce a craniofacial phenotype.  
502 The same is true for patients with FSHD2 with mutations in DNA methyltransferase type 3B  
503 (*DNMT3B*)<sup>43</sup> who have no clinical signs of immunodeficiency, centromeric instability, and  
504 facial anomalies syndrome type 1 (ICF1 [OMIM: 242860])<sup>44</sup>, the autosomal recessive disorder  
505 associated with mutations at this locus. Within our cohort, we observed multiple family members  
506 harboring *SMCHD1* mutations with only mild dysmorphic features or anosmia, and at least one  
507 individual without any dysmorphic features. Given the epigenetic function of *SMCHD1*, it is  
508 plausible that one or more genetically-interacting loci influence susceptibility to arhinia with the  
509 proximal interactors of *SMCHD1* such as *TGIF1* and *DOK7* representing prime candidates.  
510 Disentangling these genetic mechanisms in conjunction with the biochemical consequences of  
511 *SMCHD1* missense mutations in humans and model organisms will be a critical area of further

512 study, ideally in human tissue of relevance to arhinia and FSHD2 rather than the LCLs currently  
513 available.

514

515 In conclusion, we discovered that rare variants localized to an evolutionarily constrained region  
516 of *SMCHD1* are associated with BAM and isolated arhinia. Importantly, during the course of this  
517 study we learned of an independent effort by Gordon and colleagues, who also identified  
518 *SMCHD1* missense mutations in arhinia subjects. In correspondence we have compared our  
519 subjects and determined that their study provided an additional 7 subjects that were independent  
520 of our analyses (six overlapped), bringing the total to 45 arhinia subjects, (87%) of whom  
521 harbored a rare missense mutation in *SMCHD1*. Their analyses also confirmed the *SMCHD1*  
522 mutation to have occurred *de novo* in two of the overlapping subjects for which we did not have  
523 parental samples (M1 and AJ1). The molecular mechanism by which such mutations contribute  
524 to arhinia, and what differentiates FSHD2 and arhinia patients, remains unclear, though our  
525 functional modeling suggests that a simple, single locus mechanism is unlikely. Our analyses  
526 thus emphasize yet another example in a growing list of genes in which mutations can give rise  
527 to pleiotropic phenotypes across the spectrum of human anomalies. For *SMCHD1*, these  
528 phenotypes – a rare muscle disease and now, a severe craniofacial and reproductive disorder - are  
529 perplexingly diverse. Dissecting the genetic and epigenetic factors that determine phenotypic  
530 manifestations will inform both our understanding of the pathogenesis of the arhinia-BAM-  
531 FSHD spectra and, more broadly, the genetic and epigenetic architecture of oligogenic disorders.

532

533

534

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550

551 **COMPETING FINANCIAL INTERESTS**

552 The authors declare no competing financial interests.

553

554 **AUTHOR CONTRIBUTIONS**

555 M.E.T., D.R.F., E.E.D., N.K., P.J., N.D.S., and H.B. designed the study. N.D.S., L.P., K.A.W.,  
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564 which was revised and approved by all co-authors.

565

#### 566 **SUPPLEMENTARY INFORMATION**

567 **Supplementary material is available online and contains Supplementary Figs. 1-8 and**  
568 **Supplementary Tables 1-4.**

569



570

571 **Table 1. *SMCHD1* mutations observed in arhinia cohort**

Chr	Nucleotide change	Exon	Inheritance (Sample ID)	# Subjects	Sample IDs	AA Change	Gender (Sample ID)
18	c.2666926T>C	3	N/A	1	K1	p.L107P	F
18	c.2666992T>A	3	N/A	1	D1	p.M129K	M
18	c.2667009A>T	3	De Novo (AF1) N/A (M1)	2	M1,AF1	p.S135C	F
18	c.2667010G>A	3	De Novo (I1) N/A *(R1)	2	I1, R1	p.S135N	F(R1), M(I1)
18	c.2667014A>C	3	Father*	1	T1	p.E136D	M
18	c.2667016G>A	3	N/A	1	AG1	p.G137E	F
18	c.2667021A>C	3	De Novo (A1) N/A (Y1)	2	A1,Y1	p.N139H	F(A1,Y1)
18	c.2667029G>C	3	N/A	3	C1,E1,S1	p.L141F	F(S1), M(C1,E1)
18	c.2667029G>T	3	De Novo	1	V1	p.L141F	M
18	c.2674017 T>G	5	N/A*	1	AB1	p.F171V	M
18	c.2688478C>G	6	De Novo	1	AA1	p. A242G	M
18	c.2694685A>G	8	Mother*	2	O1, O4**	p.Q345R	F
18	c.2697032A>G	9	De Novo (X1,AC1,AE1) N/A (F1,L1,N1,Z1)	7	F1,L1,N1,Z1 X1,AC1,AE1	p.H348R	F (L1,X1), M(F1,N1,Z1, AC1,AE1)
18	c.2697896A>T	10	Father*	1	AH1	p.Q400L	F
18	c.2697956A>T	10	De Novo	1	P1	p.D420V	M
18	c.2700611G>C	11	N/A	1	W1	p. E473Q	M
18	c.2700837C>A	12	N/A	2	J1,U1	p.T523K	F(U1), M(J1)
18	c.2700840A>G	12	N/A	1	B1	p.N524S	M
18	c. 2703697G>A	13	N/A	1	AJ1	p.R552Q	M

572 \*Multiplex family

573 \*\*Siblings

574 A rare missense mutation was not identified in *SMCHD1* in subjects G1, H1, H2, Q1, AD1, or  
575 AI.

576 N/A = parental samples not available; AA = amino acid; M = male; F = female.

577

578 Amino acid codes: A=Ala, R=Arg, N=Asn, D=Asp, C=Cys, Q=Gln, E=Glu, G=Gly, H=His,  
579 L=Leu, M=Met, F=Phe, P=Pro, S=Ser, T=Thr, V=Val.

580

581

582 **FIGURE LEGENDS**

583

584 **Figure 1. Phenotypic spectra associated with arhinia**

585 Five representative subjects (a-e) demonstrating complete congenital arhinia and variable ocular  
586 phenotypes: a) Subject V1 (age 2) with left-sided iris coloboma b) Subject AC1 (age 10) has left-  
587 sided microphthalmia and bilateral nasolacrimal duct stenosis c) Subject U1 (as a newborn) has  
588 normal eye anatomy and vision, d) Subject O4 (age 16) has right-sided microphthalmia e)  
589 Subject A1 (young child, age unknown) has bilateral colobomatous microphthalmia, cataracts,  
590 and nasolacrimal duct atresia. f-j) All craniofacial radiographic images are from subject V1: f)  
591 Surface rendering reconstruction from a MRI 3D T1 weighted sequence showing complete  
592 absence of the nose (arrow1) g) 3D volume rendering technique (VRT) reconstruction from  
593 spiral CT showing complete absence of nasal bones (arrow) h) Coronal reconstruction from CT  
594 showing absence of nasal septal structures. The maxilla articulates with the nasal process of the  
595 frontal bone (arrow) i) Coronal MRI T2 weighted sequence showing absence of the olfactory  
596 bulb and olfactory sulcus (arrow) j) Midline MRI sagittal T1 weighted sequence. There is a high-  
597 arched palate (cleft not visible on this image) and decreased distance between the oral cavity and  
598 the anterior cranial fossa (black arrow). The rudimentary nasopharynx (\*) is blind and air-filled.  
599 The pituitary gland (white arrow) appears normal.

600

601 **Figure 2. Association analyses for rare mutation burden in arhinia**

602 Manhattan plot and quantile-quantile (q-q) plot demonstrating the significant accumulation of  
603 rare *SMCHD1* mutations in subjects with arhinia compared to the ExAC cohort ( $p = 2.9e-17$ ).  
604 Analyses involved a variant count at each gene for arhinia subjects compared to ExAC controls  
605 ( $n = 60,706$ ) who presumably do not have arhinia after filtering for allele frequency ( $MAF <$

606 0.1%), quality (mean depth  $\geq 10$ ; mapping quality  $\geq 10$ ) and predicted function (nonsynonymous,  
607 splice site, and frameshift mutations). Any gene with at least one mutation passing these criteria  
608 was included in the analysis (n = 22,445 genes). Genome-wide significance threshold was  $p <$   
609  $2.2 \times 10^{-6}$  following Bonferroni correction (red line) and only *SMCHD1* achieved this threshold.

610

611

### 612 **Figure 3. Arhinia mutations occur near the 5' GHKL-type ATPase domain**

613 a) The distribution of arhinia mutations across *SMCHD1* is tightly clustered between exons 3-12  
614 of the gene compared to b) the distribution of variants observed in *FSHD2* subjects and c) ExAC  
615 controls. *FSHD2* subjects were taken from the Leiden Open Variation Database (LOVD 3.0)<sup>45</sup>.  
616 Constraint analysis as described by Daly and colleagues<sup>10</sup> revealed that while the gene displays  
617 significant overall intolerance to deleterious mutations ( $p = 0.016$ ), this significance is almost  
618 entirely driven by constraint across the first 19 exons of *SMCHD1* ( $X^2 = 37.73$ ;  $p = 8.12 \times 10^{-10}$ ),  
619 which includes the GHKL-type ATPase domain, whereas the region from exons 20-48 are not  
620 constrained ( $X^2 = 0.87$ ;  $p = 0.35$ ). Figures were modified from the cBioPortal Mutation Mapper  
621 software v1.0.1 ([http://www.cbioportal.org/mutation\\_mapper.jsp](http://www.cbioportal.org/mutation_mapper.jsp))<sup>46,47</sup>

622

### 623 **Figure 4. DNA methylation analysis of D4Z4 repeats**

624 a) Bisulfite sequencing (BSS) of the chromosome 4q and 10q D4Z4 repeats identifies DNA  
625 hypomethylation consistent with dominant *SMCHD1* hypomorphic mutations found in *FSHD2*  
626 patients. A total of 52 CpGs were analyzed, arranged linearly from left to right, for 12 clones  
627 arranged top to bottom, each representing an independent chromosome analyzed. Each predicted  
628 CpG is represented by a box, with red boxes indicating methylated CpGs and blue boxes  
629 indicating unmethylated CpGs. b) Cartoon of the chromosome 4q and 10q D4Z4 macrosatellites

630 that vary in repeat units (RU) from 1 to ~120 RUs. The region analyzed by BSS in each RU is  
631 indicated by a green bar. \*FSHD2 requires a mutation in *SMCHD1* combined with at least 1  
632 chromosome 4q D4Z4 array ranging in size between 11-28 RUs and a permissive A-type 4q  
633 subtelomere. c) BSS observed 75% of arhinia probands with *SMCHD1* mutations that could be  
634 tested due to available material had D4Z4 hypomethylation characteristic of FSHD2, while the  
635 single proband tested without a *SMCHD1* mutation showed a normal methylation pattern. BSS  
636 was measured from the lowest quartile as previously described<sup>21</sup> and a methylation rate of <25%  
637 was considered consistent with hypomethylation observed in FSHD2. See **Supplementary**  
638 **Table 4** for further details on individual methylation status.

639

640 **Figure 5. *In vivo* modeling of *smchd1* in zebrafish demonstrates craniofacial and GnRH**  
641 **phenotypes relevant to congenital arhinia**

642 a.) Suppression of *smchd1* results in altered cartilage structures in 3 day post-fertilization (dpf) -  
643 *1.4coll1a1:egfp* larvae. Representative ventral images; *smchd1* morphants and F0 mutant larvae  
644 display a reduction in the size of the ethmoid plate (ep, as measured with solid white arrows);  
645 and abnormal jaw structures including a broadened ceratohyal angle (ch, dashed white line), and  
646 reduction in the number of ceratobranchial arches (cb, asterisks). Scale bar, 200 $\mu$ m b)  
647 Quantification of ethmoid plate width measured on ventral images. The furthest distal width (a,  
648 left panel a) was normalized to the width at the ethmoid plate-trabecula junction (b, left panel a).  
649 c) Loss of *smchd1* results in a decreased eye size; lateral bright-field images of representative  
650 3dpf control, morphant, and CRISPR/Cas9 larvae are shown. Scale bar = 300 $\mu$ m. d)  
651 Quantification of eye size area in larval batches (indicated with dashed white circle in panel c).  
652 e) Immunostaining of gonadotropin releasing hormone (GnRH) neurons in 1.5 dpf embryos with

653 a pan-GnRH antibody shows shorter terminal nerve (tn) projections from the olfactory bulb (ob)  
654 in *smchd1* models. Representative ventral views are shown; h, hypothalamus; scale bar, 100 $\mu$ m;  
655 Dashed white boxes are zoomed to show tn projections in the insets; dashed white lines (insets)  
656 indicate tn length measurement starting proximal to the ob, and extending to the tip of the tn. f)  
657 *In vivo* complementation assay of missense *SMCHD1* variants using GnRH tn length as a  
658 phenotypic readout. S135C, L141F, and H348R are recurrent mutations in arhinia cases; P690S  
659 is associated with FSHD2<sup>18</sup> V708I (rs2276092) is a common variant in ExAC and is a negative  
660 control for the assay. Orientation indicated (panels a and e) with arrows pointing to anterior (A),  
661 posterior (P), left (L) and right (R). Statistical significance is indicated with \*\*\* (p<0.0001), \*\*  
662 (p<0.01), or \*(p<0.05); g, guide RNA; NS, not significant. n=19-50 embryos/injection (panel b);  
663 n=28-59 embryos/injection (panel d); n=18-20 embryos/injection (panel f) with masked scoring;  
664 all experiments were repeated. Error bars indicate standard error of the mean.

665

### 666 **Figure 6: SMCHD1 protein modeling.**

667 Protein modeling predicts that arhinia mutations were more likely to occur on the surface of  
668 *Smchd1* and disrupt a binding surface compared to the distribution of FSHD2 mutations. A)  
669 Homology model of the N-terminal region of SMCHD1 generated with Phyre2<sup>32</sup> with residues  
670 mutated in arhinia (red) and FSHD2 (blue). All of the top 20 structural templates had GHKL  
671 domains: 16 were Hsp90 structures, two were mismatch repair proteins (MutL/Mlh1) and two  
672 were type II topoisomerases. Only those residues modeled with high confidence are shown (115-  
673 295; 314-439; 458-491; 504-535; 552-573). B) Comparison of predicted relative solvent  
674 accessibility values for residues in the N-terminal region of SMCHD1 mutated in arhinia and  
675 FSHD2. Three different predictive methods were used: NetsurfP<sup>48</sup>, I-TASSER<sup>49</sup> and SPIDER<sup>50</sup>.

676 Residues mutated in both disorders (136-137) are excluded in this analysis. P-values are  
677 calculated with the Wilcoxon rank-sum test. Boxes represent quartile distributions.

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## ONLINE METHODS

682 **Research Subject Enrollment.** We collected existing DNA or blood samples from 38 subjects  
683 with arhinia (22 male, 16 female). Whenever possible, DNA was also collected from family  
684 members. Phenotypic information was obtained via questionnaires completed by patients,  
685 parents, or referring physicians and confirmed by review of official medical records and  
686 consultation with the referring physician. Note that reproductive axis dysfunction could not be  
687 determined in pre-pubertal girls or in pre-pubertal boys without congenital microphallus or  
688 cryptorchidism. All research was approved by the Institutional Review Board of Partners  
689 Healthcare and a subset of families consented to publication of photographs (**Figure 1**).

690  
691 **Whole-Exome Sequencing (WES).** We performed WES on 26 total probands with arhinia (22  
692 in initial round and 4 that failed targeted sequencing) and 12 family members. The majority of  
693 subjects (n = 29) were sequenced at the Broad Institute (Cambridge, MA, USA),  
694 including 21 independent subjects and 1 set of affected siblings from a consanguineous family.  
695 We also sequenced 6 unaffected available family members from these subjects at the Broad  
696 Institute (families A, D, E; see **Supplementary Fig. 1**). We collected another two sporadic  
697 subjects, one trio (family V) and a mother-proband pair (family U), that had previous WES  
698 sequencing from the University of Zurich (Zurich, Zurich, Switzerland). We also collected a trio  
699 (family T) that had previously undergone WES by GeneDx (Gaithersburg, MD, USA) and  
700 contained an affected proband who also had a deceased great aunt with arhinia and coloboma.  
701 We finally received exome results for a subject (AJ1) with arhinia from the Department of  
702 Human Genetics at Nagasaki University. All exomes except sample AJ1 were aligned in house  
703 with BWA-MEM v.0.7.10 to GRCh37 and underwent joint variant calling by GATK<sup>51</sup> following  
704 best practice methods<sup>52,53</sup>. Familial relationships were confirmed by KING v1.4<sup>54</sup> and variants  
705 were annotated with Annovar v.2016-02-01<sup>55</sup> against the refseq annotation of the genome  
706 (<http://www.ncbi.nlm.nih.gov/refseq/>).

707  
708 **Whole-Genome Sequencing (WGS).** We obtained samples from 4 members of  
709 multigenerational family O<sup>6,7</sup> (see Supplementary Fig.1) and performed whole-genome deep  
710 WGS to 30X average coverage on the Illumina X Ten platform. Family O had multiple  
711 individuals with craniofacial abnormalities beyond the proband's arhinia, including a deceased  
712 maternal-half aunt with arhinia, a sister with arhinia, a mother with anosmia and subtle nasal and  
713 dental anomalies, and a maternal grandmother with mild nasal and dental anomalies. Note that  
714 samples from the affected sister, unaffected brother, and unaffected maternal half-aunt were  
715 obtained after WGS had been completed and were therefore screened for the p.Q345R variant by  
716 targeted sequencing. Variants were aligned with BWA-MEM v.7.7 to GRCh37 and GATK was  
717 used to call single nucleotide variants (SNVs) as described above.

718

719 **Genetic Association Analyses.** We compared the genic burden of rare, nonsynonymous  
 720 variants detected by WES in independent arhinia subjects from our cohort (n = 29; one affected  
 721 subject [brother] selected from consanguineous sibship) with WES data from over 60,706  
 722 controls in the the Exome Aggregation Consortium<sup>8,9</sup> (ExAC; <http://exac.broadinstitute.org/>).  
 723 Analyses were restricted to include variants that passed the following criteria: 1) high quality  
 724 (GATK Filter=PASS), 2) rare (ExAC minor allele frequency [MAF] < 0.1%), 3) mean depth ≥  
 725 10 reads, 4) a mapping quality ≥ 10, and 5) predicted to be nonsynonymous, to alter splicing, or  
 726 to cause a frameshift. As there was no gender bias among our arhinia subjects to suggest sex-  
 727 linkage (42% female), and we could not ascertain gender from the ExAC database, analyses were  
 728 restricted to autosomes. Counts between ExAC and the arhinia cohort were compared by a Fisher  
 729 exact test. Results were visualized as a Manhattan and QQ plot created by the R package  
 730 qqman<sup>56</sup>.

731  
 732 **Targeted Sequencing.** Variants of interest, as determined by our WES and WGS gene  
 733 association analysis, were subsequently confirmed by Sanger sequencing in all subjects except  
 734 T1, as DNA was not available (we are getting DNA). Analyses of these subjects demonstrated a  
 735 significant aggregation of rare mutations in *SMCHD1* restricted to exons 3, 8-10, 12, and 13. We  
 736 therefore performed targeted sequencing of these exons in all additional subjects (n = 12) using  
 737 the primers below and subjects that failed this targeted sequencing (n=4) were sent for WES as  
 738 described above.

739

<b>Exon</b>	<b>Primer Sequence 5'-3'</b>
Exon 3 fwd	TGCTTACAGGTAGATGATTGGG
Exon 3 rev	GGAATGGGATACGTAATCAGG
Exon 6&7 fwd	TTAACACTGAATACAAGTGCAATG
Exon 6&7 rev	TTCATACTTTCAAGTTAAGTTCTGTCC
Exon 8 fwd	TGTATTGGGCCAGTTTCCTC
Exon 8 rev	CCTGTGCCTCAAATAATGCTC
Exon 9 fwd	AAATGCTTAATAAAGTGCTTGATACC
Exon 9 rev	TTTATTATCCTGAGTCATTTGGAAC
Exon 10 fwd	TGTCCTTCAGCTCTGATTTGC
Exon 10 rev	GAGAAGACAAGGGAACATATAAAGG
Exon 11 fwd	TGTGTTTGTTCATTATTTCTCACA
Exon 11 rev	GGAGGAGTACACCAGTCAAAGC
Exon 12 fwd	CAGCTAGAGGGAAAAGGCCT
Exon 12 rev	TGTGAACACTTGACTGCTCA
Exon 13 fwd	GGTAATGCATTTGTTTGAAATATCC
Exon 13 rev	CTTCATGAAATGTGAGAATGGG

740

741

742 **Inheritance Testing:** For samples with a predicted de novo variant without WES we confirmed  
 743 familial relationships by determining repeat length of 10 STS markers (d15s205, d12s78,  
 744 d4s402, d13s170, d4s414, d22s283, d13s159, d2s337, d3s1267, d12s86). Inheritance of markers  
 745 was checked in each proband and proper parental inheritance was confirmed in all cases.

746 Inheritance for a single proband (P1) was confirmed in a similar manner at the University of  
747 Edinburgh with the following nine markers: cfstr1, d7s480, dxs1214, amel, nr2e3\_22, d4s2366,  
748 ilcahd, d5s629, d5s823.

749  
750 **Transcriptome Sequencing (RNAseq).** Total RNA of ~1 million cells was extracted  
751 from EBV-transformed lymphoblastoid cell line (LCLs) using TRIzol® (Invitrogen) followed by  
752 RNeasy® Mini Kit (Qiagen) column purification. RNAseq libraries were prepared using the  
753 Illumina TruSeq kit and manufacturer's instructions, as described<sup>57,58</sup>. Libraries were  
754 multiplexed, pooled and sequenced on multiple lanes of an Illumina HiSeq2500, generating an  
755 average of 33 million paired-end reads of 76 bp. Quality checking of sequence reads was  
756 assessed by fastQC (v. 0.10.1) (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>).  
757 Next, sequence reads were aligned to human reference genome Ensembl GRCh37 (v. 71) using  
758 GSNAP (v. 12-19-2014) at its default parameter setting<sup>59</sup>. Quality checking of alignments was  
759 assessed by a custom script utilizing Picard Tools (<http://broadinstitute.github.io/picard/>),  
760 RNASEQC<sup>60</sup>, RSeQC<sup>61</sup> and SamTools<sup>62</sup>. Gene level counts were tabulated using BedTools's  
761 multibamcov algorithm (v. 2.17.0)<sup>63</sup> on unique alignments for each library at all Ensembl genes  
762 (GRCh37 v.71). We found the threshold to detect expressed genes to be at least six uniquely  
763 mapped reads by relying on analysis of External RNA Controls Consortium (ERCC) spike-ins as  
764 we have previously described<sup>57</sup>. After filtering out short genes (transcript lengths < 250 nt) and  
765 rRNA and tRNA genes, only the 15,936 genes that met the detection threshold in all case  
766 samples or all control samples were kept for further analysis. To account for the effect of the  
767 covariance among family members, a generalized linear-mixed model (GLMM) approach was  
768 used. For this task, a mixed model package, lme4 (v. 1.1.10)<sup>64</sup> was employed in R (v. 3.2.2).  
769 Specifically, gene-level expression data across samples as raw counts was fitted to a following  
770 GLMM based on a Poisson-lognormal approach  $condition + (1|familyId) + (1|obsId)$ , where  
771 *condition* is a fixed factor that describes a binary disease status of an individual, *familyId* is a  
772 random factor that accounts for similarity in expression due to shared genetic background and  
773 *obsId* is a random factor that accounts for individual-level random effects. This model converged  
774 on 15,478 genes. An evolutionary constrained gene list was retrieved from the ExAC database  
775 (v. 0.3 release 3-16-2015), where constrained genes were defined to be those with a probability  
776 of being intolerant to loss of function mutations  $\geq 0.9$ . A protein-protein interaction network of  
777 differentially expressed genes (nominal  $p < 0.05$ ) was constructed based on physical interaction  
778 data from the BioGRID database (v 3.4.135)<sup>65</sup>. The resulting network contained 1,069 proteins  
779 and 2,593 pair-wise interactions in which a protein had 4.86 connections (degrees) on average.  
780 We defined hub proteins to be in the top 5th percentile of degree distribution in this network,  
781 which corresponds to 17 connections or more.

782  
783 **Western Blot:** Protein was harvested from 1 million LCLs in 23 total subjects: 10 subjects with  
784 arhinia harboring presumably pathogenic *SMCHD1* variants, 11 unaffected family members  
785 without *SMCHD1* mutations, and two family members with a mutation in *SMCHD1* and anosmia  
786 or a hypoplastic nose (AH3 and AH5, respectively; **Supplemental Fig. 9**). Protein extraction  
787 was performed with the following procedure: 1) Cells were washed in 1x PBS and lysed in 300  
788 ul ice-cold 1 x RIPA buffer ([http://www.bio-world.com/productinfo/4\\_62\\_465/7465/RIPA-Buffer-X-pH.html](http://www.bio-world.com/productinfo/4_62_465/7465/RIPA-Buffer-X-pH.html)) supplemented with 5 mM PMSF. 2) After 30 min. incubation on ice, cell  
789 lysates were cleared by centrifugation (15G, 15 min., 4°C) and soluble proteins concentration  
790 was assayed with BCA reagent



792 (<https://www.thermofisher.com/order/catalog/product/23225#/23225>). Extracted proteins  
793 (15-30 ul/sample) were next separated by a 8% sodium dodecyl sulfate polyacrylamide gel  
794 electrophoresis (SDS-PAGE; Bio-Rad MiniProtean 3 Cell, 2 hr 15 mA) and transferred onto a  
795 polyvinylidene fluoride (PVDF) membrane (Bio-Rad cat#1620174) using liquid transfer system  
796 (Bio-Rad Ready Gel Cell) at 4°C, 10V for 16 hrs. Western blotting was performed using two  
797 sets of SMCHD1 antibodies: 1) Bethyl Laboratories A302-872A-M (anti-SMCHD1, C-  
798 terminus); 2) Abcam ab122555 (anti-SMCHD1, N-terminus). We used two loading control  
799 antibodies: 1) Abcam ab6046 (beta-Tubulin load control) 2) Abcam ab8227 (beta-Actin load  
800 control). Antibody dilutions were used as recommended by manufacturer. Primary antibodies  
801 were diluted in tris-buffered saline and tween 20 (TBST) buffer and 1% BSA, secondary HRP-  
802 conjugated antibody (1:20,000 dilution) in TBST without BSA. Membrane was cut alongside 75  
803 kDa marker (BioRad Precision Plus Protein standards cat# 161-0375) and the upper part was  
804 used for blotting SMCHD1 (MW=250 kDa), while the lower part for blotting beta-Tubulin  
805 (MW=50 kDa) and beta-Actin (MW=42 kDa) controls. Blotting with primary antibody was  
806 carried out overnight at 4°C on a rocking platform, followed by three 10 min. washes in TBST at  
807 room temperature. Blotting with secondary antibody was carried out at room temperature for 1  
808 hr, followed by three 10 min. washes in TBST. Re-blotting of SMCHD1 with an alternative  
809 antibody, the previously used primary antibody was stripped off with mild stripping buffer, as  
810 described: <http://www.abcam.com/ps/pdf/protocols/stripping%20for%20reprobing.pdf>. Western  
811 blot were luminesced with ECL reagent (Bio-Rad cat# 170-5060) and developed with the  
812 ChemiDoc MP system ([http://www.bio-rad.com/en-us/product/chemidoc-imaging-  
813 systems/chemidoc-mp-system](http://www.bio-rad.com/en-us/product/chemidoc-imaging-systems/chemidoc-mp-system)). Automated protein quantification was done using Image Lab  
814 5.2.1 software (BioRad).

815  
816  
817 **CRISPR/Cas9 Genome Editing in Mouse Embryos.** To generate mouse embryos carrying the  
818 p.Leu141Phe disease associated missense variant in *Smchd1*, a double stranded DNA oligomer  
819 (CCTTTGCGTAAGTAACCTGCTC) that provides a template for the guide RNA sequence was  
820 cloned into px461. The full gRNA template sequence is amplified from the resulting px461 clone  
821 using universal reverse primer and T7 tagged forward primers. The guide RNA PCR template is  
822 used for *in vitro* RNA synthesis using T7 RNA polymerase (Neb), and the RNA template is  
823 subsequently purified using RNeasy mini kit (Qiagen) purification columns. Cas9 mRNA was  
824 procured from Tebu Bioscience. The wild-type and mutant repair templates (chr17:71,463,705-  
825 71,463,818 GRCm38) are synthesized as 114bp ultramers bearing the desired sequence change  
826 from IDT. The injection mix contains Cas9 mRNA (50ng/ul), guide RNA (25ng/ul) and repair  
827 template DNA (150ng/ul). Injections are performed in mouse zygotes and the embryos are later  
828 harvested for analysis at 11.5 and 13.5 dpc stage of embryonic development.

829  
830 **Optical Projection Tomography.** Whole mouse embryos were mounted in 1% agarose,  
831 dehydrated in methanol and then cleared overnight in BABB (1 part Benzyl Alcohol: 2 parts  
832 Benzyl Benzoate). The sample was then imaged using a Bioptonic OPT Scanner 3001  
833 (Bioptonic, UK) using tissue autofluorescence (excitation 425nm/emmission 475nm) to capture  
834 the anatomy. The resulting images were reconstructed using Bioptonic proprietary software,  
835 automatically thresholded and merged to a single 3D image output using Bioptonic Viewer  
836 software.

837

838 **DNA methylation analysis.** The DNA methylation status of the D4Z4 region was assayed as  
839 previously described<sup>21</sup>. Bisulfite conversion was performed on 1 µg of genomic DNA using the  
840 EpiTect Bisulfite Kit (Qiagen) per manufacturer's instructions, and 200 ng of converted genomic  
841 DNA was used for PCR. Bisulfite sequencing (BSS) analysis of 52 CpGs in the *DUX4* promoter  
842 region of the 4q and 10q D4Z4 repeats was performed using primers BSS167F:  
843 TTTTGGGTTGGGTGGAGATTTT and BSS1036R: AACACCR TACCRAACTTACACCCTT,  
844 followed by nested PCR with BSS475F: TTAGGAGGGAGGGAGGGAGGTAG and  
845 BSS1036R using 10% of the first PCR product. PCR products were cloned into the pGEM-T  
846 Easy vector (Promega), sequenced, and analyzed using web-based analysis software BISMA  
847 (<http://biochem.jacobs-university.de/BDPC/BISMA/>)<sup>66</sup> with the default parameters. Standard  
848 genomic PCR was performed on non-converted DNA to identify the 4qA, 4qA-L and 4qB  
849 chromosome<sup>67</sup>. Specific 4q and 10q haplotypes were identified and assigned as previously  
850 described<sup>68,69</sup>. The presence of the *DUX4* polyadenylation site was determined by BS-PCR as  
851 previously described<sup>42</sup>.

852  
853 **Determination of 4q35 and 10q26 D4Z4 array sizes.** Peripheral blood leukocytes were  
854 embedded in agarose plugs and digested with three different restriction enzymes (EcoRI,  
855 EcoRI/BlnI, and XapI). Restriction fragments were separated by pulse field gel electrophoresis  
856 (PFGE) and sized and visualized by Southern blot with a p13E-11 probe, and in some subjects, a  
857 D4Z4 probe for confirmation<sup>70</sup>.

858  
859 **Gene suppression and *in vivo* complementation of zebrafish embryos.** Splice blocking  
860 morpholinos (MO)s targeting the *Danio rerio smchd1* exon 3 splice donor (e3i3; 5'-  
861 AGGTGTGATTT CAGACTTACGCAAC-3') or exon 5 splice donor (e5i5; 5'-  
862 TGATTATGAAGACCGCACCTTTGAA-3') were designed and synthesized by Gene Tools  
863 LLC (Philomath, Oregon). To determine the optimal MO dose for *in vivo* complementation  
864 studies, we injected increasing doses (3 ng, 6 ng, and 9 ng of each MO; 1 nl MO injected per  
865 embryo; 1-2 cell stage) into *-1.4coll1a1:egfp*<sup>71</sup> embryos harvested from natural mating of  
866 heterozygous transgenic adults maintained on an AB background. To determine MO efficiency,  
867 we used Trizol (ThermoFisher) to extract total RNA from embryos at 1 day post-fertilization  
868 (dpf) according to manufacturer's instructions. Resulting total RNA was reverse transcribed into  
869 cDNA using the Superscript III Reverse Transcriptase kit (ThermoFisher), and was used as  
870 template in RT-PCR reactions to amplify regions flanking MO target sites. RT-PCR products  
871 were gel-purified using the QIAquick gel extraction kit (Qiagen), cloned (TOPO-TA;  
872 Invitrogen), and plasmid purified from individual colonies was Sanger sequenced according to  
873 standard protocols to identify the precise alteration of endogenous transcript. For rescue  
874 experiments, a wild-type (WT) human *SMCHD1* ORF (NM\_015295) construct was obtained  
875 commercially (OriGene Technologies) and subcloned into the pCS2+ vector. Point mutations  
876 were introduced into pCS2+ vectors as described<sup>72</sup> and all vectors were sequence confirmed. WT  
877 and variant *SMCHD1* constructs were linearized with *NotI*, and mRNA was transcribed using the  
878 mMessage mMachin e kit SP6 transcription kit (ThermoFisher). Unless otherwise noted, 9 ng  
879 MO (either e3i3 or e5i5) was used in parallel or in combination with 25 pg *SMCHD1* mRNA for  
880 *in vivo* complementation studies.

881  
882

883 **CRISPR/Cas9 genome editing in zebrafish embryos.** We used CHOPCHOP  
884 (<http://chopchop.cbu.uib.no/>) to identify a guide (g)RNA targeting sequence within the smchd1  
885 coding regions (5' GAGATGTCGAAAGTCCGCGG 3'). Guide RNAs were in vitro transcribed  
886 using the GeneArt precision gRNA synthesis kit (ThermoFisher) according to manufacturer's  
887 instructions. Zebrafish embryos were obtained from -1.4coll1a1:egfp embryos harvested from  
888 natural mating of heterozygous transgenic adults maintained on an AB background; 1 nl of  
889 injection cocktail containing 100 pg/nl gRNA and 200 pg/nl Cas9 protein (PNA Bio) were  
890 injected into the cell of embryos at the one-cell stage. To determine targeting efficiency in  
891 founder (F0) mutants, we extracted genomic DNA from 2 dpf embryos and PCR-amplified the  
892 region flanking the gRNA target site. PCR products were denatured, reannealed slowly and  
893 separated on a 15% TBE 1.0 mm precast polyacrylamide gel; it was incubated in ethidium  
894 bromide and imaged on a ChemiDoc system (BioRad) to visualize hetero/homoduplexes. To  
895 estimate the percent mosaicism of smchd1 F0 mutants (n=5), PCR products were gel purified  
896 (Qiagen), and cloned into a TOPO-TA vector (ThermoFisher). Plasmid was prepped from  
897 individual colonies (n=10-12 colonies/embryo), and Sanger sequenced according to standard  
898 procedures.

899  
900 **Phenotypic analyses in zebrafish.** To study craniofacial structures (cartilage or eye  
901 development), larval batches were reared at 28<sup>0</sup>C and imaged live at 3 dpf using the Vertebrate  
902 Automated Screening Technology Bioimager (VAST; software version 1.2.2.8; Union  
903 Biometrica) mounted on an AxioScope A1 (Zeiss) microscope using an AxioCam 503  
904 monochromatic camera and Zen Pro 2012 software (Zeiss). Fluorescence imaging of GFP  
905 positive cells on ventrally positioned larvae was conducted as described<sup>73</sup>. In parallel, we  
906 obtained lateral bright-field images of whole larvae using the VAST onboard camera. To  
907 evaluate gonadotropin-releasing hormone (GnRH) neurons, 1.5 dpf embryos were dechorionated  
908 and fixed in a solution of 4% paraformaldehyde (PFA) and 7% picric acid for 2 hours at room  
909 temperature. Embryos were then washed with a solution of phosphate buffered saline with 0.1%  
910 Triton X-100 (PBS-T) and stored at 4<sup>0</sup>C until staining. For whole-mount immunostaining,  
911 embryos were washed briefly with 0.1% trypsin in PBS; washed in PBS-T; and dehydrated at -  
912 20<sup>0</sup>C in pre-chilled 100% acetone for 15 min. Next, embryos were washed in PBS-T; blocked in  
913 a solution of 2% BSA, 1% DMSO, 0.5% Triton-X100, and 5% calf serum for 1 hour at room  
914 temperature. We used rabbit anti-GnRH antibody (1:500 dilution; Sigma) for primary detection.  
915 Following overnight incubation of primary antibody, we washed with blocking solution, and  
916 incubated with AlexaFluor 555 anti-rabbit secondary antibody (1:500; ThermoFisher) for 2 hours  
917 at room temperature. Images were acquired manually with an AxioZoom.V16 microscope and  
918 AxioCam 503 monochromatic camera, and were z-stacked using Zen Pro 2012 software (Zeiss).  
919 Cartilage structure, eye area, and GnRH neuron projection length was measured using ImageJ  
920 software (NIH); pairwise comparisons to determine statistical significance were calculated using  
921 a student's t-test. For ceratobranchial pair counts, we used a  $\chi^2$  test to determine statistical  
922 significance. All experiments were repeated at least twice.

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## 930 REFERENCES

- 931 1. Bosma, J.F., Henkin, R.I., Christiansen, R.L. & Herdt, J.R. Hypoplasia of the nose and  
932 eyes, hyposmia, hypogeusia, and hypogonadotrophic hypogonadism in two males. *J*  
933 *Craniofac Genet Dev Biol* **1**, 153-84 (1981).
- 934 2. Hogan, B.L. *et al.* Small eyes (Sey): a homozygous lethal mutation on chromosome 2  
935 which affects the differentiation of both lens and nasal placodes in the mouse. *J Embryol*  
936 *Exp Morphol* **97**, 95-110 (1986).
- 937 3. Glaser, T. *et al.* PAX6 gene dosage effect in a family with congenital cataracts, aniridia,  
938 anophthalmia and central nervous system defects. *Nat Genet* **7**, 463-71 (1994).
- 939 4. Schmidt-Sidor, B. *et al.* Malformations of the brain in two fetuses with a compound  
940 heterozygosity for two PAX6 mutations. *Folia Neuropathol* **47**, 372-82 (2009).
- 941 5. Solomon, B.D. *et al.* Compound heterozygosity for mutations in PAX6 in a patient with  
942 complex brain anomaly, neonatal diabetes mellitus, and microphthalmia. *Am J Med*  
943 *Genet A* **149A**, 2543-6 (2009).
- 944 6. Cho, C.-H., Shakibaei, M., Merker, H.-J. & Klein, M. The rare malformation of nasal  
945 aplasia. *Mund-, Kiefer- und Gesichtschirurgie* **10**, 107-118 (2006).
- 946 7. Thiele, H., Musil, A., Nagel, F. & Majewski, F. Familial arhinia, choanal atresia, and  
947 microphthalmia. *Am J Med Genet* **63**, 310-3 (1996).
- 948 8. Lek, M. *et al.* Analysis of protein-coding genetic variation in 60,706 humans. *Nature*  
949 **536**, 285-91 (2016).
- 950 9. Lek, M. *et al.* Analysis of protein-coding genetic variation in 60,706 humans. *bioRxiv*  
951 (2015).
- 952 10. Samocha, K.E. *et al.* A framework for the interpretation of de novo mutation in human  
953 disease. *Nat Genet* **46**, 944-50 (2014).
- 954 11. Chen, K. *et al.* The epigenetic regulator Smchd1 contains a functional GHKL-type  
955 ATPase domain. *Biochem J* (2016).
- 956 12. Blewitt, M.E. *et al.* SmcHD1, containing a structural-maintenance-of-chromosomes  
957 hinge domain, has a critical role in X inactivation. *Nat Genet* **40**, 663-9 (2008).
- 958 13. Chen, K. *et al.* Genome-wide binding and mechanistic analyses of Smchd1-mediated  
959 epigenetic regulation. *Proc Natl Acad Sci U S A* **112**, E3535-44 (2015).
- 960 14. Gendrel, A.V. *et al.* Epigenetic functions of smchd1 repress gene clusters on the inactive  
961 X chromosome and on autosomes. *Mol Cell Biol* **33**, 3150-65 (2013).
- 962 15. Mould, A.W. *et al.* Smchd1 regulates a subset of autosomal genes subject to monoallelic  
963 expression in addition to being critical for X inactivation. *Epigenetics Chromatin* **6**, 19  
964 (2013).
- 965 16. Lemmers, R.J. *et al.* Digenic inheritance of an SMCHD1 mutation and an FSHD-  
966 permissive D4Z4 allele causes facioscapulohumeral muscular dystrophy type 2. *Nat*  
967 *Genet* **44**, 1370-4 (2012).
- 968 17. Hirano, T. At the heart of the chromosome: SMC proteins in action. *Nat Rev Mol Cell*  
969 *Biol* **7**, 311-22 (2006).
- 970 18. Lemmers, R.J. *et al.* Inter-individual differences in CpG methylation at D4Z4 correlate  
971 with clinical variability in FSHD1 and FSHD2. *Hum Mol Genet* **24**, 659-69 (2015).
- 972 19. van den Boogaard, M.L. *et al.* Double SMCHD1 variants in FSHD2: the synergistic  
973 effect of two SMCHD1 variants on D4Z4 hypomethylation and disease penetrance in  
974 FSHD2. *Eur J Hum Genet* **24**, 78-85 (2016).

- 975 20. Lemmers, R.J. *et al.* Hemizygoty for SMCHD1 in Facioscapulohumeral Muscular  
976 Dystrophy Type 2: Consequences for 18p Deletion Syndrome. *Hum Mutat* **36**, 679-83  
977 (2015).
- 978 21. Jones, T.I. *et al.* Identifying diagnostic DNA methylation profiles for  
979 facioscapulohumeral muscular dystrophy in blood and saliva using bisulfite sequencing.  
980 *Clin Epigenetics* **6**, 23 (2014).
- 981 22. Jones, T.I. *et al.* Individual epigenetic status of the pathogenic D4Z4 macrosatellite  
982 correlates with disease in facioscapulohumeral muscular dystrophy. *Clin Epigenetics* **7**,  
983 37 (2015).
- 984 23. Steven, C. *et al.* Molecular characterization of the GnRH system in zebrafish (*Danio*  
985 *rerio*): cloning of chicken GnRH-II, adult brain expression patterns and pituitary content  
986 of salmon GnRH and chicken GnRH-II. *Gen Comp Endocrinol* **133**, 27-37 (2003).
- 987 24. Liu, C. *et al.* A secreted WNT-ligand-binding domain of FZD5 generated by a frameshift  
988 mutation causes autosomal dominant coloboma. *Hum Mol Genet* **25**, 1382-91 (2016).
- 989 25. Chassaing, N. *et al.* Targeted resequencing identifies PTCH1 as a major contributor to  
990 ocular developmental anomalies and extends the SOX2 regulatory network. *Genome Res*  
991 **26**, 474-85 (2016).
- 992 26. Yahyavi, M. *et al.* ALDH1A3 loss of function causes bilateral  
993 anophthalmia/microphthalmia and hypoplasia of the optic nerve and optic chiasm. *Hum*  
994 *Mol Genet* **22**, 3250-8 (2013).
- 995 27. Whitlock, K.E., Illing, N., Brideau, N.J., Smith, K.M. & Twomey, S. Development of  
996 GnRH cells: Setting the stage for puberty. *Mol Cell Endocrinol* **254-255**, 39-50 (2006).
- 997 28. Zohar, Y., Munoz-Cueto, J.A., Elizur, A. & Kah, O. Neuroendocrinology of reproduction  
998 in teleost fish. *Gen Comp Endocrinol* **165**, 438-55 (2010).
- 999 29. Abraham, E., Palevitch, O., Gothilf, Y. & Zohar, Y. The zebrafish as a model system for  
1000 forebrain GnRH neuronal development. *Gen Comp Endocrinol* **164**, 151-60 (2009).
- 1001 30. Sharpe, J. *et al.* Optical projection tomography as a tool for 3D microscopy and gene  
1002 expression studies. *Science* **296**, 541-5 (2002).
- 1003 31. Blewitt, M.E. *et al.* An N-ethyl-N-nitrosourea screen for genes involved in variegation in  
1004 the mouse. *Proc Natl Acad Sci U S A* **102**, 7629-34 (2005).
- 1005 32. Kelley, L.A., Mezulis, S., Yates, C.M., Wass, M.N. & Sternberg, M.J. The Phyre2 web  
1006 portal for protein modeling, prediction and analysis. *Nat Protoc* **10**, 845-58 (2015).
- 1007 33. Chen, J., Bardes, E.E., Aronow, B.J. & Jegga, A.G. ToppGene Suite for gene list  
1008 enrichment analysis and candidate gene prioritization. *Nucleic Acids Res* **37**, W305-11  
1009 (2009).
- 1010 34. Hall, J.G. Pena-Shokeir phenotype (fetal akinesia deformation sequence) revisited. *Birth*  
1011 *Defects Res A Clin Mol Teratol* **85**, 677-94 (2009).
- 1012 35. Solomon, B.D., Gropman, A. & Muenke, M. Holoprosencephaly Overview. in  
1013 *GeneReviews(R)* (eds. Pagon, R.A. *et al.*) (Seattle (WA), 1993).
- 1014 36. Lederer, D. *et al.* Deletion of KDM6A, a histone demethylase interacting with MLL2, in  
1015 three patients with Kabuki syndrome. *Am J Hum Genet* **90**, 119-24 (2012).
- 1016 37. Lindgren, A.M. *et al.* Haploinsufficiency of KDM6A is associated with severe  
1017 psychomotor retardation, global growth restriction, seizures and cleft palate. *Hum Genet*  
1018 **132**, 537-52 (2013).
- 1019 38. de Greef, J.C. *et al.* Clinical features of facioscapulohumeral muscular dystrophy 2.  
1020 *Neurology* **75**, 1548-54 (2010).

- 1021 39. van Deutekom, J.C. *et al.* Evidence for subtelomeric exchange of 3.3 kb tandemly  
1022 repeated units between chromosomes 4q35 and 10q26: implications for genetic  
1023 counselling and etiology of FSHD1. *Hum Mol Genet* **5**, 1997-2003 (1996).
- 1024 40. Clapp, J. *et al.* Evolutionary conservation of a coding function for D4Z4, the tandem  
1025 DNA repeat mutated in facioscapulohumeral muscular dystrophy. *Am J Hum Genet* **81**,  
1026 264-79 (2007).
- 1027 41. Mitsuhashi, H., Mitsuhashi, S., Lynn-Jones, T., Kawahara, G. & Kunkel, L.M.  
1028 Expression of DUX4 in zebrafish development recapitulates facioscapulohumeral  
1029 muscular dystrophy. *Hum Mol Genet* **22**, 568-77 (2013).
- 1030 42. Calandra, P. *et al.* Allele-specific DNA hypomethylation characterises FSHD1 and  
1031 FSHD2. *J Med Genet* **53**, 348-55 (2016).
- 1032 43. van den Boogaard, M.L. *et al.* Mutations in DNMT3B Modify Epigenetic Repression of  
1033 the D4Z4 Repeat and the Penetrance of Facioscapulohumeral Dystrophy. *Am J Hum*  
1034 *Genet* **98**, 1020-9 (2016).
- 1035 44. Weemaes, C.M. *et al.* Heterogeneous clinical presentation in ICF syndrome: correlation  
1036 with underlying gene defects. *Eur J Hum Genet* **21**, 1219-25 (2013).
- 1037 45. Fokkema, I.F. *et al.* LOVD v.2.0: the next generation in gene variant databases. *Hum*  
1038 *Mutat* **32**, 557-63 (2011).
- 1039 46. Cerami, E. *et al.* The cBio cancer genomics portal: an open platform for exploring  
1040 multidimensional cancer genomics data. *Cancer Discov* **2**, 401-4 (2012).
- 1041 47. Gao, J. *et al.* Integrative analysis of complex cancer genomics and clinical profiles using  
1042 the cBioPortal. *Sci Signal* **6**, p11 (2013).
- 1043 48. Petersen, B., Petersen, T.N., Andersen, P., Nielsen, M. & Lundegaard, C. A generic  
1044 method for assignment of reliability scores applied to solvent accessibility predictions.  
1045 *BMC Struct Biol* **9**, 51 (2009).
- 1046 49. Yang, J. *et al.* The I-TASSER Suite: protein structure and function prediction. *Nat*  
1047 *Methods* **12**, 7-8 (2015).
- 1048 50. Heffernan, R. *et al.* Improving prediction of secondary structure, local backbone angles,  
1049 and solvent accessible surface area of proteins by iterative deep learning. *Sci Rep* **5**,  
1050 11476 (2015).
- 1051 51. Miller, D.T. *et al.* Consensus statement: chromosomal microarray is a first-tier clinical  
1052 diagnostic test for individuals with developmental disabilities or congenital anomalies.  
1053 *Am J Hum Genet* **86**, 749-64 (2010).
- 1054 52. DePristo, M.A. *et al.* A framework for variation discovery and genotyping using next-  
1055 generation DNA sequencing data. *Nat Genet* **43**, 491-8 (2011).
- 1056 53. Van der Auwera, G.A. *et al.* From FastQ data to high confidence variant calls: the  
1057 Genome Analysis Toolkit best practices pipeline. *Curr Protoc Bioinformatics* **43**, 11 10  
1058 1-33 (2013).
- 1059 54. Manichaikul, A. *et al.* Robust relationship inference in genome-wide association studies.  
1060 *Bioinformatics* **26**, 2867-73 (2010).
- 1061 55. Wang, K., Li, M. & Hakonarson, H. ANNOVAR: functional annotation of genetic  
1062 variants from high-throughput sequencing data. *Nucleic Acids Res* **38**, e164 (2010).
- 1063 56. Turner, S.D. qqman: an R package for visualizing GWAS results using Q-Q and  
1064 manhattan plots. *bioRxiv* (2014).
- 1065 57. Blumenthal, I. *et al.* Transcriptional consequences of 16p11.2 deletion and duplication in  
1066 mouse cortex and multiplex autism families. *Am J Hum Genet* **94**, 870-83 (2014).

- 1067 58. Sugathan, A. *et al.* CHD8 regulates neurodevelopmental pathways associated with autism  
1068 spectrum disorder in neural progenitors. *Proc Natl Acad Sci U S A* **111**, E4468-77 (2014).
- 1069 59. Wu, T.D. & Nacu, S. Fast and SNP-tolerant detection of complex variants and splicing in  
1070 short reads. *Bioinformatics* **26**, 873-81 (2010).
- 1071 60. DeLuca, D.S. *et al.* RNA-SeQC: RNA-seq metrics for quality control and process  
1072 optimization. *Bioinformatics* **28**, 1530-2 (2012).
- 1073 61. Wang, L., Wang, S. & Li, W. RSeQC: quality control of RNA-seq experiments.  
1074 *Bioinformatics* **28**, 2184-5 (2012).
- 1075 62. Li, H. *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**,  
1076 2078-9 (2009).
- 1077 63. Quinlan, A.R. & Hall, I.M. BEDTools: a flexible suite of utilities for comparing genomic  
1078 features. *Bioinformatics* **26**, 841-2 (2010).
- 1079 64. Bates, D., Mächler, M., Bolker, B. & Walker, S. Fitting Linear Mixed-Effects Models  
1080 Using lme4. *2015* **67**, 48 (2015).
- 1081 65. Stark, C. *et al.* BioGRID: a general repository for interaction datasets. *Nucleic Acids Res*  
1082 **34**, D535-9 (2006).
- 1083 66. Rohde, C., Zhang, Y., Reinhardt, R. & Jeltsch, A. BISMA--fast and accurate bisulfite  
1084 sequencing data analysis of individual clones from unique and repetitive sequences. *BMC*  
1085 *Bioinformatics* **11**, 230 (2010).
- 1086 67. Lemmers, R.J. *et al.* A unifying genetic model for facioscapulohumeral muscular  
1087 dystrophy. *Science* **329**, 1650-3 (2010).
- 1088 68. Lemmers, R.J. *et al.* Specific sequence variations within the 4q35 region are associated  
1089 with facioscapulohumeral muscular dystrophy. *Am J Hum Genet* **81**, 884-94 (2007).
- 1090 69. Lemmers, R.J. *et al.* Worldwide population analysis of the 4q and 10q subtelomeres  
1091 identifies only four discrete interchromosomal sequence transfers in human evolution.  
1092 *Am J Hum Genet* **86**, 364-77 (2010).
- 1093 70. Lemmers, R.J., O'Shea, S., Padberg, G.W., Lunt, P.W. & van der Maarel, S.M. Best  
1094 practice guidelines on genetic diagnostics of Facioscapulohumeral muscular dystrophy:  
1095 workshop 9th June 2010, LUMC, Leiden, The Netherlands. *Neuromuscul Disord* **22**,  
1096 463-70 (2012).
- 1097 71. Kague, E. *et al.* Skeletogenic fate of zebrafish cranial and trunk neural crest. *PLoS One* **7**,  
1098 e47394 (2012).
- 1099 72. Niederriter, A.R. *et al.* In vivo modeling of the morbid human genome using Danio rerio.  
1100 *J Vis Exp*, e50338 (2013).
- 1101 73. Isrie, M. *et al.* Mutations in Either TUBB or MAPRE2 Cause Circumferential Skin  
1102 Creases Kunze Type. *Am J Hum Genet* **97**, 790-800 (2015).
- 1103  
1104  
1105  
1106