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

Citation for published version:

Shaw, ND, Brand, H, Kupchinsky, ZA, Bengani, H, Plummer, L, Jones, TI, Erdin, S, Williamson, KA, Rainger, J, Stortchevoi, A, Samocha, K, Currall, BB, Dunican, DS, Collins, RL, Willer, JR, Lek, A, Lek, M, Nassan, M, Pereira, S, Kammin, T, Lucente, D, Silva, A, Seabra, CM, Chiang, C, An, Y, Ansari, M, Rainger, JK, Joss, S, Smith, JC, Lippincott, MF, Singh, SS, Patel, N, Jing, JW, Law, JR, Ferraro, N, Verloes, A, Rauch, A, Steindl, K, Zweier, M, Scheer, I, Sato, D, Okamoto, N, Jacobsen, C, Tryggestad, J, Chernausek, S, Schimmenti, LA, Brasseur, B, Cesaretti, C, García-Ortiz, JE, Buitrago, TP, Silva, OP, Hoffman, JD, Mühlbauer, W, Ruprecht, KW, Loeys, BL, Shino, M, Kaindl, AM, Cho, C-H, Morton, CC, Meehan, RR, van Heyningen, V, Liao, EC, Balasubramanian, R, Hall, JE, Seminara, SB, Macarthur, D, Moore, SA, Yoshiura, K, Gusella, JF, Marsh, JA, Graham Jr, JM, Lin, AE, Katsanis, N, Jones, PL, Crowley Jr, WF, Davis, EE, FitzPatrick, DR & Talkowski, ME 2017, 'SMCHD1 mutations associated with a rare muscular dystrophy can also cause isolated arhinia and Bosma arhinia microphthalmia syndrome', *Nature Genetics*, vol. 49, no. 2, pp. 238-248. <https://doi.org/10.1038/ng.3743>

Digital Object Identifier (DOI):

[10.1038/ng.3743](https://doi.org/10.1038/ng.3743)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Nature Genetics

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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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93 **Keywords:** constraint, methylation, pleiotropy, mutation, exome aggregation consortium, hypogonadotropic
94 hypogonadism

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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)¹. 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 mice² and cause
143 rudimentary or malformed noses in humans³⁻⁵. 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 arhinia³⁻⁵. 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)^{6,7}. 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^{8,9}. 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)¹⁰, 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 = 1.26×10^{-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 = 8.27×10^{-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 domain¹¹ and a 3' SMC-hinge domain (for dynamic DNA binding) that serves as an
240 epigenetic regulator of both autosomal and X-linked genes¹²⁻¹⁵. 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 muscle¹⁶. 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 SMCHD1¹⁷.
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)¹⁸. 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), respectively¹⁸. In both models, loss of SMCHD1 repressive

260 activity manifests as a decrease in DNA methylation at SMCHD1 binding sites^{11,16,19,20}. 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^{21,22}.
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 architecture²³. 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 studies²⁴⁻²⁶. *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 teleosts²⁷⁻²⁹.
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 FSHD2¹⁶ 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 tomography³⁰
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^{12,31}, 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 structure¹¹. We generated a structural model of the
362 N-terminal region of SMCHD1 with Phyre2³² (**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 analysis³³
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)¹³. 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)³⁴, and heterozygous loss-of-function mutations in *TGIF1* cause
409 holoprosencephaly-4 (OMIM 142946), which may include arhinia, microphthalmia, and cleft
410 palate³⁵. 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)^{36,37} 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 phenotypes¹⁸. 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**)^{16,21,38,39}. 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^{16,18,20}. 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 females¹²,
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^{40,41}.

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 known⁴². 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*)⁴³ who have no clinical signs of immunodeficiency, centromeric instability, and
504 facial anomalies syndrome type 1 (ICF1 [OMIM: 242860])⁴⁴, 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

535 **ACKNOWLEDGMENTS**

536 We thank all participants, family members, and clinical staff for their generous contributions of
537 time and materials to this research. We thank Tammy Gillis, Jayla Ruliera, and Mary Anne
538 Anderson for technical assistance. This project was funded by grants from the National Institutes
539 of Health (R00MH095867 to M.E.T., P01GM061354 to M.E.T., J.F.G., C.C.M., and E.C.L.,
540 T32HD007396 to H.B., R01HD081256 to M.E.T., P50HD028138 to W.F.C, S.B.S., M.E.T.,
541 N.K., and E.E.D., K23HD073304-02 and 1S12ES025429-01 to N.D.S., R01AR062587 to P.L.J.),
542 the March of Dimes (FY15-255 to M.E.T.), the Medical Research Council (MR/M02122X/1 to
543 J.A.M.), the German Research Foundation (SFB665 to A.M.K), the Berlin Institute of Health
544 (BIH-CRG1 to A.M.K.), D.R.F, He.B, K.A.W, J.R, J.K.R and J.A.M are funded by program
545 grants from the Medical Research Council (MRC) Human Genetics Unit award to the University
546 of Edinburgh. MA is funded by the University of Edinburgh Institute of Genomics and
547 Molecular Medicine Translational Initiative Fund. N.K. is a distinguished Jean and George
548 Brumley Professor, and M.E.T. is supported as the Desmond and Ann Heathwood MGH
549 Research Scholar.

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.,
556 M.N., S.P., T.K., D.L., A.S., S.J., J.C.S., M.F.L., S.S., N.P., J.L., N.F., A.V., A.R., K.S., I.S.,
557 D.S., N.O., C.J., J.T., S.C., L.A.S., B.B., C.C., J.E.G., T.P.B., O.P.S., J.D.H., W.M., K.W.R.,

558 B.L., M.S., A.K., C.H.C., C.C.M., V.V.H., R.B., J.E.H., S.B.S, J.M.G., A.E.L., W.F.C., D.R.F.,
559 recruited patients and collected clinical information and samples. Z.A.K, He.B., L.P., S.E, T.I.J.,
560 J.R.W, C.G, A.S., C.M.S, Y.A, B.B.C., M.A., J.K.R, M.Z., J.W.J., E.L., S.A.M., N.K, P.L.J,
561 E.E.D, D.R.F performed molecular genetic and animal modeling studies, H.B., K.S., R.L.C, A.L,
562 M.L, J.F.G, D.G.M, M.E.T performed genomic analyses, J.M. performed protein
563 modeling. N.D.S., H.B., N.K., J.F.G., P.L.J., E.E.D, D.R.F., and M.E.T. wrote the manuscript,
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 ≥ 10 ; mapping quality ≥ 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 2.2×10^{-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)⁴⁵.
616 Constraint analysis as described by Daly and colleagues¹⁰ 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)^{46,47}

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 described²¹ 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 μ 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 μ 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, 100µm;
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 FSHD2¹⁸ 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 Phyre2³² 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: NetsurfP⁴⁸, I-TASSER⁴⁹ and SPIDER⁵⁰.

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.

678
679

680 ONLINE METHODS

681

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 GATK⁵¹ following
704 best practice methods^{52,53}. Familial relationships were confirmed by KING v1.4⁵⁴ and variants
705 were annotated with Annovar v.2016-02-01⁵⁵ 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^{6,7} (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^{8,9} (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 qqman⁵⁶.

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

Exon	Primer Sequence 5'-3'
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	TGTGTTTGTTCATTATTTCTCAC
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^{57,58}. 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 setting⁵⁹. Quality checking of alignments was
759 assessed by a custom script utilizing Picard Tools (<http://broadinstitute.github.io/picard/>),
760 RNASEQC⁶⁰, RSeQC⁶¹ and SamTools⁶². Gene level counts were tabulated using BedTools's
761 multibamcov algorithm (v. 2.17.0)⁶³ 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 described⁵⁷. 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)⁶⁴ 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 ≥ 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)⁶⁵. 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) 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 Bioptonics OPT Scanner 3001
833 (Bioptonics, UK) using tissue autofluorescence (excitation 425nm/emmission 475nm) to capture
834 the anatomy. The resulting images were reconstructed using Bioptonics proprietary software,
835 automatically thresholded and merged to a single 3D image output using Bioptonics Viewer
836 software.

837

838 **DNA methylation analysis.** The DNA methylation status of the D4Z4 region was assayed as
839 previously described²¹. 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/>)⁶⁶ with the default parameters. Standard
848 genomic PCR was performed on non-converted DNA to identify the 4qA, 4qA-L and 4qB
849 chromosome⁶⁷. Specific 4q and 10q haplotypes were identified and assigned as previously
850 described^{68,69}. The presence of the *DUX4* polyadenylation site was determined by BS-PCR as
851 previously described⁴².

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 confirmation⁷⁰.

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*⁷¹ 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 described⁷² and all vectors were sequence confirmed. WT
877 and variant *SMCHD1* constructs were linearized with *NotI*, and mRNA was transcribed using the
878 mMessage mMachine 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 28⁰C 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 described⁷³. 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 4⁰C 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 20⁰C 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 χ^2 test to determine statistical
922 significance. All experiments were repeated at least twice.

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