# Recurrent *de novo* missense variants across multiple histone H4 genes underlie a neurodevelopmental syndrome

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**Summary**

Chromatin is essentially an array of nucleosomes, each of which consists of the DNA double-stranded fiber wrapped around a histone octamer. This organization supports cellular processes like DNA replication, DNA transcription and DNA repair in all eukaryotes. Human histone H4 is encoded by fourteen canonical histone H4 genes, all differing at the nucleotide level but encoding an invariant protein. Here we present a cohort of 29 subjects with *de novo* missense variants in six H4 genes (*H4C3*, *H4C4, H4C5, H4C6, H4C9* and *H4C11*) identified by whole exome sequencing and matchmaking. All individuals present with neurodevelopmental features of intellectual disability and motor and/or gross developmental delay, while non-neurological features are more variable. Ten amino acids are affected, six of which recurrently, and are all located within the H4 core or C-terminal tail. These variants cluster to specific regions of the core H4 globular domain, where protein-protein interactions occur with either other histone subunits or histone chaperones. Functional consequences of the identified variants were evaluated in zebrafish embryos, which displayed abnormal general development, defective head organs and reduced body axis length, providing compelling evidence for the causality of the reported disorder(s). While multiple

developmental syndromes have been linked to chromatin-associated factors, missense-bearing histone variants (e.g. H3 oncohistones) are only recently emerging as a major cause of pathogenicity. Our findings establish a broader involvement of H4 variants in developmental syndromes.

Histones are amongst the most slowly evolving genes in eukaryotes.[1,2] Histone H4 acts as a functional unit by forming a dimer with H3, but unlike H3 there are no variant isoforms linked to specific cellular processes. We previously reported four individuals with a primordial dwarfism phenotype with *de novo* missense variants affecting Lys91 in *H4C3*(*HIST1H4C*; MIM 602827) and *H4C11*(*HIST1H4J*; MIM 602826), a critical residue near the C-terminus of the protein prone to post-translational modifications (PTMs) such as acetylation[3], or more important here, ubiquitination.[4-6] Somatic variation in H4 is potentially relevant in a cancer setting,[7,8] although described variation impacts multiple residues in contrast to the highly recurrent pathogenic variants observed for the established oncohistone, H3.[8] This could be reconducted to the fact that while the functional importance of PTMs on N-tails of H3 is linked to the chromatin effectors they correlate to[9-11], variants affecting the histone core (H4 in this case) have more direct and global consequences on chromatin organization, as they impact nucleosome structure and dynamics.[12]

We have identified an additional cohort of 29 individuals with *de novo* missense variants in six different histone H4 genes: *H4C3* (*HIST1H4C*), *H4C4* (*HIST1H4D*; MIM 602823), *H4C5* (*HIST1H4E*; MIM 602830), *H4C6* (*HIST1H4F*; MIM 602824), *H4C9* (*HIST1H4I*; MIM 602833) and *H4C11 (HIST1H4J)* (Figure 1A, Table 1). The cohort was collected using trio-based whole-exome sequencing in combination with data sharing via Genematcher[13] and DECIPHER.[14] This study received ethical approval from the New Zealand Health and Disability Ethics Committee (16/STH/3) and London–Riverside REC (09/H0706/20). All

families provided consent to be involved in this project, with separate consent obtained for the use of photos.

There are fourteen canonical *histone H4* genes in the human genome, clustering in three genomic loci. At a nucleotide level, all genes are different, but together they encode an identical protein. Transcription of these genes is independently regulated, with differing expression levels observed during brain development[15,16] and in human tissues.[4] The genes harboring variants identified in our cohort are amongst the more highly expressed, however as H4 transcripts are not polyadenylated and therefore missed in most RNAseq protocols, limited expression data is available.

All variants observed were absent from control databases (1KG, gnomAD v2.1.1).[17] Furthermore, there were no missense variants affecting Lys91 in any of the fourteen canonical histone H4 genes in gnomAD, and only extremely rarely were substitutions observed for the other positions in different H4 genes. The absence of any substitutions at Lys91 could reflect a stronger requirement for fidelity at this position, especially given the post-translation modifications of Lys91.[18]

The genetic findings of the cohort are striking, especially given that histones are some of the slowest evolving eukaryotic proteins and human H4 is 92% conserved with the yeast orthologue.[1,2] In the human population, the histone H4 genes are tolerant to both loss-of-function and missense variation (gnomAD). We identify nine sites across the 103 amino acid protein with a mutation, six of which were found recurrently (Pro32, Arg40, Arg45, His75, Lys91 and Tyr98), including two where the same site (Pro32 and Arg40) is mutated in multiple different H4 genes. All sites are conserved through to *Saccharomyces cerevisiae*. The mutated residues cluster in two main regions of histone H4 (Figure 1B); one cluster centers on the first α-helix of H4 (Figure 1B; purple spheres), a region important for DNA contacts and protein interactions with H3 and histone chaperones.[18] Arg45 is positioned in the loop following the first α-helix and forms one of the sprockets of the nucleosome which

contacts the minor groove of DNA. Substitutions at Arg45 in *Saccharomyces cerevisiae* have proven to be deleterious to growth and fitness with altered chromatin remodeling.[7,19,20] The second cluster is within the core of the nucleosome (Figure 1B, orange spheres), where important structural contacts exist between the H3-H4 dimer and with histone chaperones.[18] Suggestive evidence for these other variants also originates from *S. cerevisiae*, where a Gly94 mutant (a key residue for H4 C-terminal flexibility) confers reduced viability,[21] whereas a His75 mutant disrupts DNA damage repair processes.[22] Other studied variants in similar regions (either somatic 'oncohistone' variants in H4,[7,8] or recently described germline variants in H3[23]) are predicted to perturb either nucleosome stability or interaction with histone chaperones, suggesting the H4 variants described in our cohort likely cause similar effects. These complementary observations, alongside the significant recurrence of specific variants, provide strong evidence for pathogenicity.

All individuals displayed intellectual disability (ID) and the majority demonstrated global and/or gross motor developmental delay (Table 2, Table S1). Other neurodevelopmental features such as hypotonia (34%), seizures (17%) or autism (17%) were present in some individuals but less common. Brain MRI was generally normal except for two individuals with delayed myelination. Microcephaly was commonly observed (Figure 2A), with an occipitofrontal circumference Z-score smaller than -2 SD evident in 16% individuals at birth and becoming progressively more severe with age (76% at the most recent exam). Short stature (defined as Z-score for height smaller than -2 standard deviations (SD)) and failure to thrive were common features (38%), however without significant change over time (Figure 2A). Clustering of anthropometric data by H4 gene or protein region revealed no obvious genotype-phenotype patterns (Figure 2A). The age range of the cohort is between 10 months and 52 years. Interestingly, the oldest individual shows signs of premature aging with greyed, thinning hair and wrinkly skin, looking at least two decades beyond his biological age, which didn't occur in his parents. Premature aging has also been observed in Rahman syndrome (MIM 617537), caused by pathogenic variants in *H1-4(HIST1H1E*; MIM

142220).[24] This phenotype appears to be milder in our H4 cohort, however the majority of the individuals are still quite young. One individual died from leukemia stemming from myelodysplasia (P28), but no other individuals were reported to have bone marrow abnormalities.

Non-neurological features were variable across the cohort. Facial features comprised a wide spectrum (Figure 2B). While some individuals were relatively non-dysmorphic, others had a common presentation affecting the facial midline, with hypertelorism (17%), a high nasal bridge (or conversely very low nasal bridge) with a broad nasal base (38%) and narrow nares, wide mouth with a gap between central incisors or other tooth anomalies (21%) (Figure 2C), and moderately pointy chin. Visual impairment such as strabismus, astigmatism, or myopia were reasonably common (61% individuals), and 24% individuals demonstrated hearing impairment. Skeletal development was normal for most individuals, however recurring features such as vertebral or digit abnormalities were present in several individuals (Figure 2D), and particularly severe in individual P28. Variability in clinical features and growth was noted even amongst the seven individuals harboring the same *de novo* variant Arg45Cys in *H4C5* (Figure 2B, Figure S1).

To validate the pathogenic effect of these variants, we expressed wild type human histone H4 and the histone H4 variants pertaining to this study in zebrafish by means of mRNA microinjection (Figure 3). The complete conservation of the zebrafish and human histone H4 proteins at the amino acid sequence level and the early, mRNA-mediated ectopic overexpression make this an optimal set-up for assaying the variants' effect on early development. Early embryonic effects were evaluated at 28 hours post fertilization (hpf) as fundamental developmental processes such as gastrulation and primary organogenesis are then completed. All variants tested displayed significant visible developmental effects compared to microinjection of the corresponding wild type H4 gene, except the Pro32Ala and Arg40Cys variants. Specifically, classification was based on cephalic development, anterior-posterior axis establishment and tissue necrosis during early embryonic

development, as these parameters captured the major defects and the cellular toxicity (Figure 3A,B,C) observed across all variants analyzed. Phenotypic observation revealed that variants affecting Lys91 and His75 caused the strongest effects. Interestingly, these two variants both cluster at the core of the nucleosome, and His75 plays a role in DNA damage repair,[22] a process previously suggested to be involved in the syndromic features of Lys91 individuals.[4] His75 is at the interface between two histone molecules within the octamer (in this case H4 and H2B) and it is located in the *lrs* (loss-of-ribosomal DNA silencing) domain of H4[22], a nucleosomal surface structure reported to have gene-specific silencing function in yeast[19]. Additionally, we observed a dosage-dependent effect for Arg40His and Arg45Cys (Figure 3D, E), which has been noted for other sprocket arginine substitutions and may relate to possible roles in higher-order organization of chromatin.[25] Interestingly, such sprocket function has been reported for both the *sin* (switch-independent) and *lrs* H4 domains containing Arg45 and His75, respectively.[26] These regions have an almost identical three-dimensional structure[26] and play a role in gene regulation (repression), the perturbation of which likely results in pathogenicity, and was detected in our functional assay. The variability in frequency of phenotype occurrence across variants may reflect the importance of the affected residues in cellular processes crucial to active cell proliferation, as the early zebrafish embryo is a system in which cells have a relatively short cell cycle time. The milder effect observed in our functional assay for variants affecting Pro32 and Arg40 may point to a moderate requirement, however the strong recurrence of variants affecting these residues in our cohort provides alternative evidence to support their pathogenicity. Altogether, these results provide a first picture of the variety of phenotypes caused by the assayed variants, supporting our genetic data. However, as their interpretation is limited by their transience and the inherent variability of our current mRNA assay, further testing in a stable model is required to get more insight in the molecular mechanisms linking genetic variants and phenotype.

Through genetic and developmental findings, we have identified pathogenic substitutions in six genes encoding histone H4 in a large cohort of individuals with a neurodevelopmental syndrome. Despite well-established cellular requirements for post-translational modification of the N-terminal tail of H4[27-29], it is notable that no *de novo* variants were identified in this region.

Recently, a large cohort of individuals with a neurodegenerative and developmental disorder were reported harbouring *de novo* missense variants in the histone H3 replication-independent genes, *H3F3A* (MIM 601128) and *H3F3B* (MIM 601058).[23] While there are phenotypic commonalities between the H3.3 cohort and individuals presented here, the presentations are distinct. The H3F3A/H3F3B cohort appears to have a more expansive neurological dysfunction with anomalies noted on imaging, accompanied by septal or genital abnormalities and craniosynostosis. In contrast, our H4 cohort has ID/DD and microcephaly as presenting features, but only more rarely are there other neurodevelopmental abnormalities. The pathogenic variants identified in *H3F3A* and *H3F3B* are located throughout the protein, with a smaller number of recurrently occurring variants. In comparison, for histone H4, the high level of recurrent variants observed is significant, along with the clear clustering of the variants in two regions of the H4 protein.

The redundancy of the H4 genes in the human genome is remarkable. Loss-of-function variants in H4 genes are present in the healthy population,[17] and are even present in homozygous form,[30] supporting our hypothesis that the variants identified here act through a dominant effect. This disease mechanism, combined with the paralogous landscape of H4 genes, presents opportunities for future treatment strategies through targeted knockdown of specific H4 gene products.

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**Author Contributions**

LSB and GvH identified and recruited the study subjects. LSB collated the clinical information. FT, LSB and GvH designed the study. KD performed molecular cloning. KK, MF

and PM performed the structural modelling. FT, JB and GvH designed zebrafish experiments. FT carried out the zebrafish experiments. All other authors assisted with genetic and clinical information for affected individuals. FT, LSB and GvH wrote the manuscript. All authors approved the final manuscript.

## Web Resources

Online Mendelian Inheritance in Man: https://omim.org/

## Data and Code Availability

Full genetic data is not available due to privacy regulations.

## Declaration of Interests

The authors declare no competing interests.

**Figure 1. H4 variants identified in the cohort. (A)** Highly recurrent variants were found in six different H4 genes (*H4C3*, *H4C4*, *H4C5*, *H4C6*, *H4C9 and H4C11*), which all encode an identical protein. Aggregate prevalence of disease-causing amino acid changes is also shown. The N-terminal methionine is cleaved from histone H4, therefore all numbering is relative to the mature polypeptide, in keeping with the protein literature. **(B)** The affected residues of H4 (orange ribbon) either cluster to the N-terminal α-helix facing towards DNA (Cluster 1, purple spheres), or are located in regions buried within the nucleosome core (Cluster 2, orange spheres). Size of sphere indicates the relative prevalence of substitutions affecting that residue.

**Figure 2. Clinical characteristics of individuals with histone H4 gene variants.**

**(A)** Individuals with variants in histone H4 genes demonstrate a reduction in height, weight and brain growth (OFC, occipitofrontal circumference), with the latter significantly progressing as the individuals age. There are no detectable genotype-phenotype patterns separating by the specific histone H4 gene or variant cluster. **** $P<0.0001$. **(B)** Facial dysmorphism affecting midline structures is noticeable amongst the cohort, but highly variable, with no obvious genotype-phenotype correlation. **(C)** Individuals can present with abnormalities in the appearance and position of teeth (for example, P5, P25). A recurring feature present in several individuals is a noticeable gap between the upper central incisors. **(D)** Individuals with variants in histone H4 genes also show a spectrum of toe anomalies, ranging from no anomalies present (for example P21) through to severe 2-3 toe (P1, P28) or 3-4 toe (P25) syndactyly, which can be bilateral. Toes can also be short (P19, P28).

**Figure 3. H4 variants induce developmental defects in zebrafish embryos. (A)** Phenotypical characterization in 28 hpf embryos. Representative images of observed phenotypes in zebrafish embryos 28 hr post-fertilization microinjected with mRNA encoding either wild-type or identified variants at the one-cell stage. The different classes are defined on general development and necrosis. **(B)** and **(C)** Detailed view of cephalic necrosis (B) and curved tail (C) phenotypes. **(D)** and **(E)** Quantification of the phenotypical classification as described in (A). Variants reported in (D) were microinjected with 50 pg/embryo, additional testing with 100 pg/embryo is reported in (E). Data marked with a hash symbol was previously published in [4]. Fisher's exact test: ns: not significant, $P>0.05$; *$P<0.05$; ****$P<0.0001$: Scale bars: (A): 100 µm; (B,C): 50 µm.

**Table 1. Variants identified in H4 genes.**

| Number of individuals | Gene | Chromosomal position (hg38) | cDNA | Protein | H4# numbering | CADD PHRED | REVEL score |
|---|---|---|---|---|---|---|---|
| 1 | *H4C3* | 6:26104044 | c.97C>G | p.(Pro33Ala) | Pro32Ala | 23.1 | 0.526 |
| 2 | *H4C3* | 6:26104045 | c.98C>T | p.(Pro33Leu) | Pro32Leu | 25.7 | 0.438 |
| 3 | *H4C3* | 6:26104221 | c.274A>C | p.(Lys92Gln) | Lys91Gln | 26.6 | 0.706 |
| 1 | *H4C4* | 6:26188955 | c.122G>A | p.(Arg41His) | Arg40His | 23.9 | 0.459 |
| 1 | *H4C5* | 6:26204739 | c.95A>C | p.(Lys32Thr) | Lys31Thr | 26.6 | 0.523 |
| 1 | *H4C5* | 6:26204742 | c.98C>G | p.(Pro33Arg) | Pro32Arg | 25.3 | 0.541 |
| 1 | *H4C5* | 6:26204750 | c.106C>T | p.(Arg36Trp) | Arg35Trp | 26.6 | 0.494 |
| 1 | *H4C5* | 6:26204757 | c.113T>C | p.(Leu38Pro) | Leu37Pro | 27.5 | 0.659 |
| 4 | *H4C5* | 6:26204765 | c.121C>T | p.(Arg41Cys) | Arg40Cys | 26.6 | 0.446 |
| 7 | *H4C5* | 6:26204780 | c.136C>T | p.(Arg46Cys) | Arg45Cys | 26.3 | 0.589 |
| 2 | *H4C5* | 6:26204939 | c.295T>C | p.(Tyr99His) | Tyr98His | 26.9 | 0.317 |
| 1 | *H4C6* | 6:26240708 | c.283G>A | p.(Gly95Arg) | Gly94Arg | 24.5 | 0.677 |
| 1 | *H4C9* | 6:27139430 | c.122G>T | p.(Arg41Leu) | Arg40Leu | 26.7 | 0.628 |
| 2 | *H4C9* | 6:27139535 | c.227A>G | p.(His76Arg) | His75Arg | 25.3 | 0.754 |
| 1 | *H4C11* | 6:27824245 | c.121C>T | p.(Arg41Cys) | Arg40Cys | 25.8 | 0.541 |

RefSeq IDs: *H4C3*: NM_003542.4, *H4C4*: NM_003539.4, *H4C5*: NM_003545.3, *H4C6*: NM_003540.4, *H4C9*: NM_003495.2, *H4C11*: NM_021968.4. # Note on Nomenclature: To refer to the residues belonging to this study, HGVS Variant nomenclature would include Methionine-1 (Met1) at the translation initiating site (e.g. H4C3 Pro3**3**Ala [c.97C>G; p.(Pro3**3**Ala)]. However, as the research field of epigenetics and oncohistones typically drops this first post-translationally removed methionine, we have also done so. Therefore, the above-mentioned example (included in this study) is referred to as H4C3 Pro3**2**Ala.

**Table 2: Clinical features of individuals in the H4 cohort**

| Clinical Feature | Proportion (percentage) |
|---|---|
| **Neurodevelopment** | |
| Intellectual Disability | 29/29 (100%) |
| Developmental Delay | 29/29 (100%) |
| Hypotonia | 10/29 (34%) |
| Seizures | 5/29 (17%) |
| Autism | 5/29 (17%) |
| Ataxia | 4/29 (14%) |
| **Growth** | |
| Microcephaly - prenatal onset | 2/19 (11%) |
| - postnatal | 20/29 (69%) |
| Short stature | 11/29 (38%) |
| Failure to thrive | 11/29 (38%) |
| **Skeletal Features** | |
| Craniosynostosis | 2/29 (7%) |
| Digit anomalies | 4/29 (14%) |
| Vertebral anomalies | 4/27 (15%) |
| **Facial features** | |
| Hypertelorism | 5/29 (17%) |
| Upslanting palpebral fissures | 3/29 (10%) |
| Broad nasal tip | 11/29 (38%) |
| Thin upper lip / vermillion | 4/29 (14%) |
| Teeth anomalies | 6/29 (21%) |
| **Other features** | |
| Recurrent infections | 4/29 (14%) |
| Visual impairment | 17/28 (61%) |
| Hearing impairment | 7/29 (24%) |
| Age range | 10m – 52 y (median 10 y 11 m) |

Y – year, m – months.

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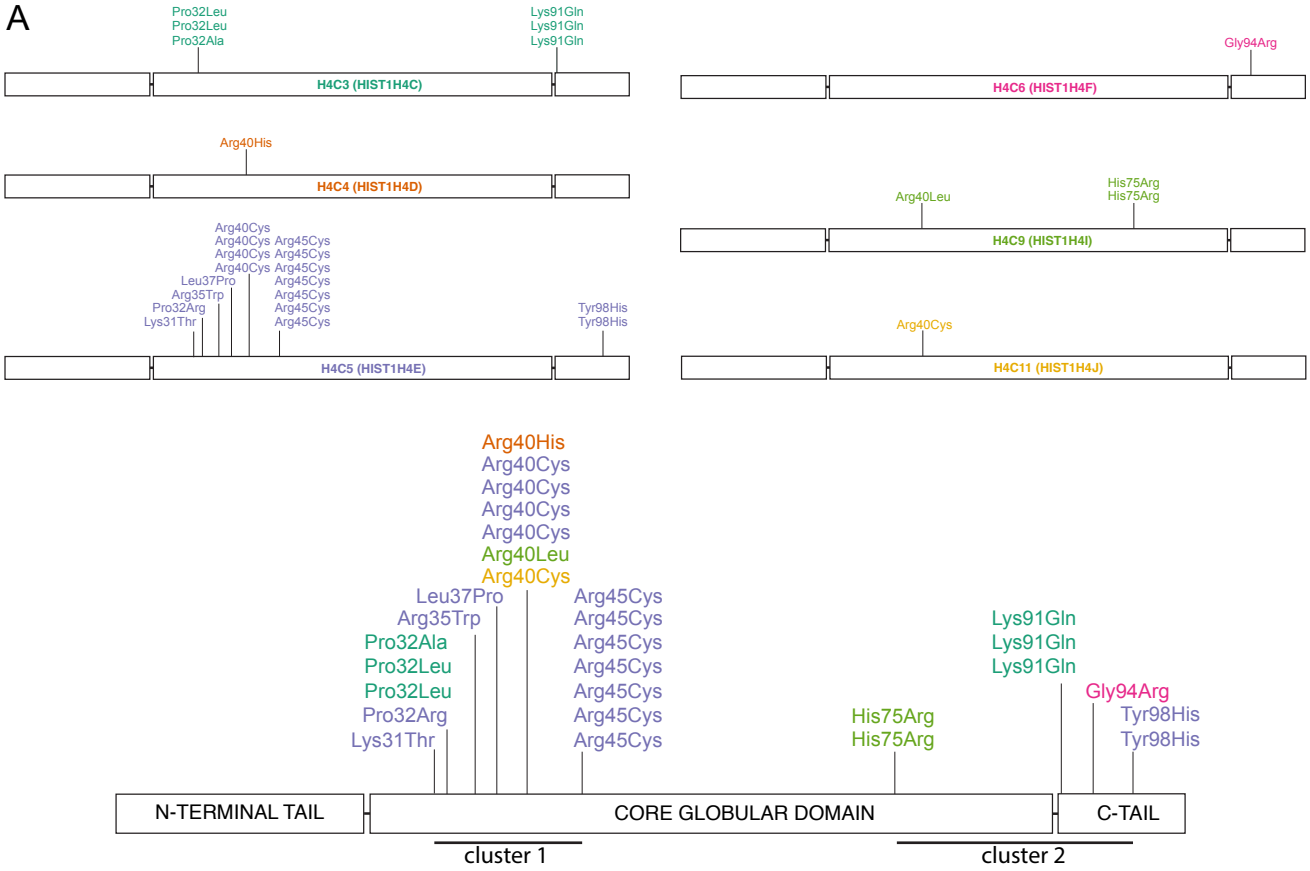
# Figure 1.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Arg40His
H4C4 (HIST1H4D)
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His75Arg
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Tyr98His
Tyr98His
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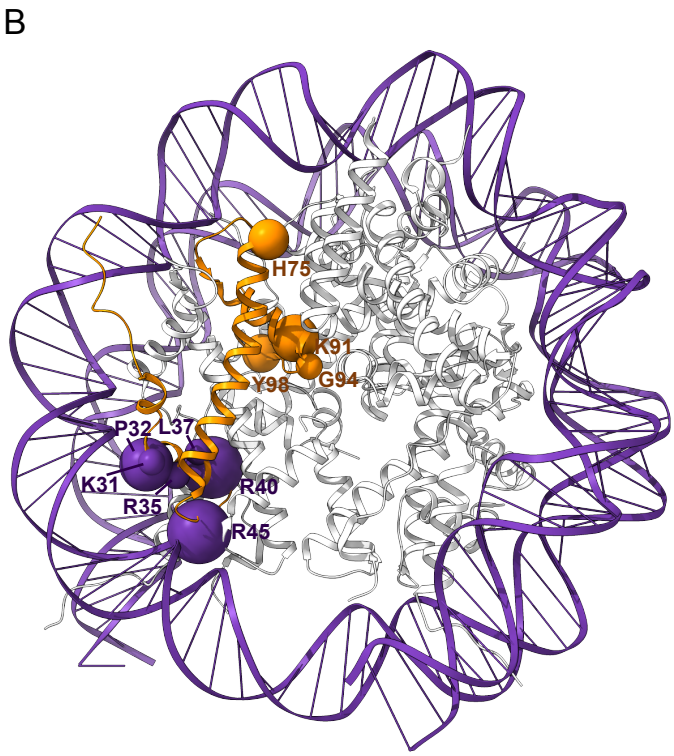
CORE GLOBULAR DOMAIN
C-TAIL
cluster 1
cluster 2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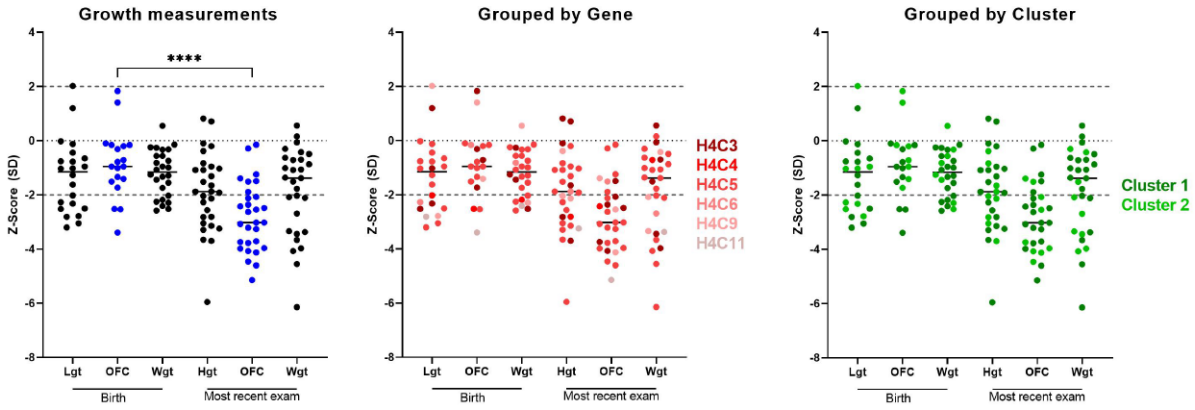
R35
R45

# Figure 2

A



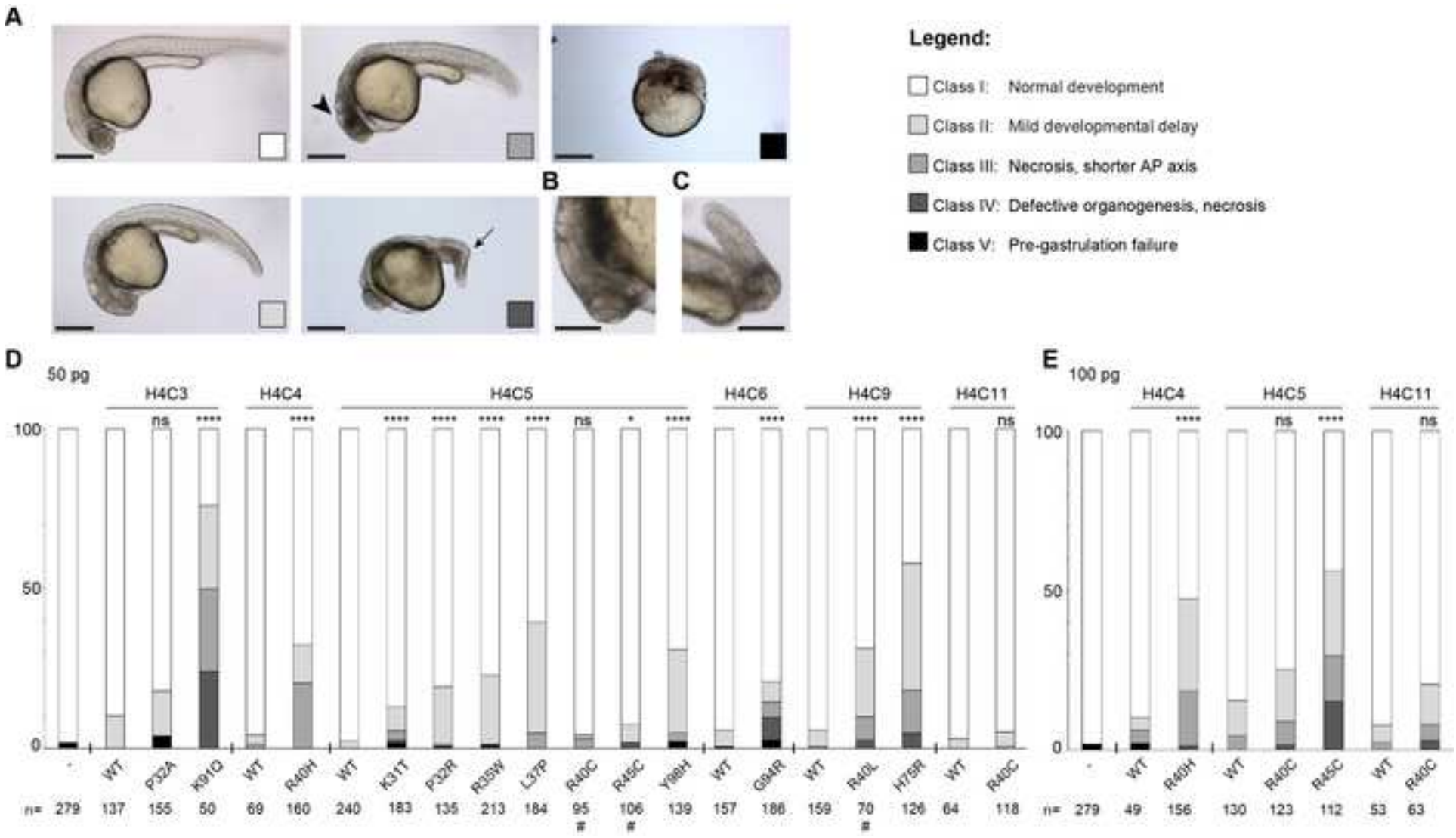

B


C


D

Figure 3





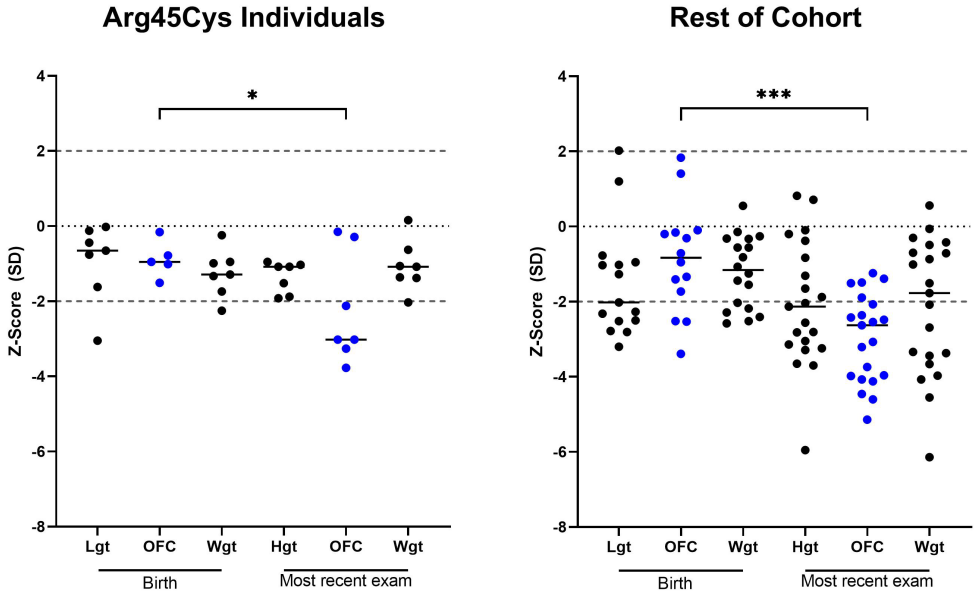



**Figure S1. Variability in growth parameters in individuals with the Arg45Cys variant.** Growth parameters of individuals with the most common recurring variant Arg45Cys highlight the variability observed across the cohort, especially in OFC changes as the individuals age. One-way ANOVA, allowing for multiple comparions;* p = 0.041, *** p = 0.0002. OFC, occipitofrontal circumference.

a


b

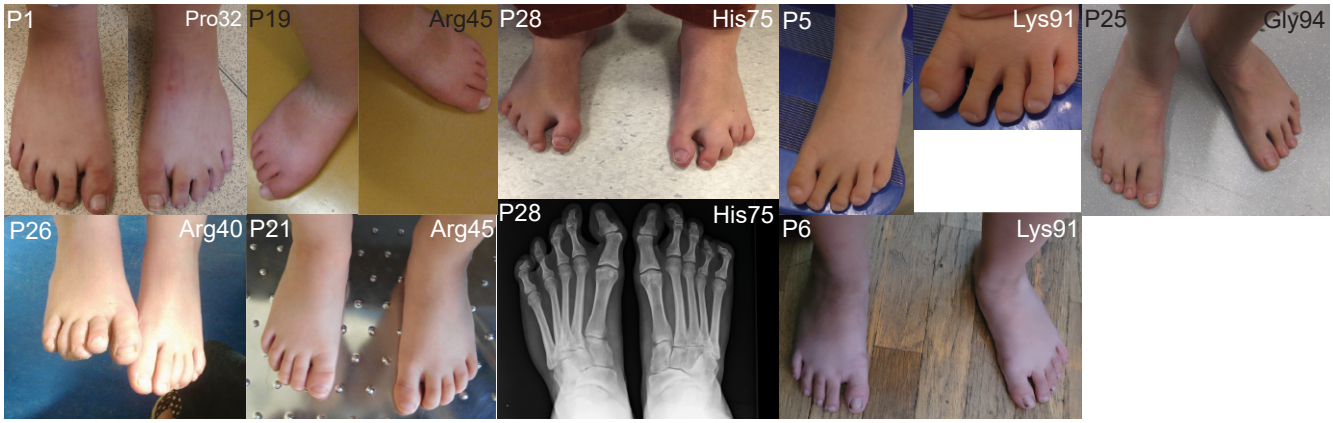



**Figure S2.** Clinical observations in the histone H4 patient cohort.
(a) Individuals can present with abnormalities in the appearance and position of teeth (for example, P5, P24). A recurring feature present in several individuals is a noticeable gap between the upper central incisors.
(b) Individuals with variants in histone H4 genes also show a spectrum of toe anomalies, ranging from no anomalies present (for example P20) through to severe 2-3 toe (P1, P27) or 3-4 toe (P24) syndactyly, which can be bilateral. Toes can also be short (P18, P27).

Table S2: Oligonucleotide sequences used for cloning and site-directed mutagenesis.

| Gene | Variant | Name | Sequence | Purpose |
|---|---|---|---|---|
| H4C3 (HIST1H4C) | WT | Hist1H4C_FL_F<br>Hist1H4C_FL_R | 5'-GCCACCATGTCTGGTCGCGGCAAAG-3'<br>5'-TCAGCCGCCGAAGCCATAC-3' | cDNA amplification |
| | Pro32Ala | Hist1H4C_P32A_F<br>Hist1H4C_P32A_R | 5'- ACATCCAGGGCATTACAAAAGCGGCTATTCGCC-3'<br>5'-GGCGAATAGCCGCTTTTGTAATGCCCTGGATGT-3' | Site-directed mutagenesis |
| H4C4 (HIST1H4D) | WT | Hist1H4D_FL_F<br>Hist1H4D_FL_R | 5'-GCCACCATGTCTGGCCGCGGTAAGGG-3'<br>5'-TCAGCCGCCGAAGCCATAAAG-3' | cDNA amplification |
| | Arg40His | Hist1H4D_R40H_F<br>Hist1H4D_R40H_R | 5'-CCTGGCTCGCCACGGCGGCGTCA-3'<br>5'-TGACGCCGCCGTGGCGAGCCAGG-3' | Site-directed mutagenesis |
| H4C5 (HIST1H4E) | WT | Hist1H4E_FL_F<br>Hist1H4E_FL_R | 5'-GCCACCATGTCTGGTCGCGGCAAAGGC-3'<br>5'-TTAGCCGCCGAAGCCGTAAAG-3' | cDNA amplification |
| | Lys31Thr | Hist1H4E_K31T_F<br>Hist1H4E_K31_R | 5'-ATAACATCCAGGGCATTACCACGCCTGCCATCC-3'<br>5'-GGATGGCAGGCGTGGTAATGCCCTGGATGTTAT-3' | Site-directed mutagenesis |
| | Pro32Arg | Hist1H4E_P32R_F<br>Hist1H4E_P32R_R | 5'-GGCATTACCAAGCGTGCCATCCGGCGC-3'<br>5'-GCGCCGGATGGCACGCTTGGTAATGCC-3' | Site-directed mutagenesis |
| | Arg35Trp | Hist1H4E_R35W_F<br>Hist1H4E_R35W_R | 5'-CAAGCCTGCCATCTGGCGCCTTGCTCG-3'<br>5'-CGAGCAAGGCGCCAGATGGCAGGCTTG-3' | Site-directed mutagenesis |
| | Leu37Pro | Hist1H4E_L37P_F<br>Hist1H4E_L37P_R | 5'-CATCCGGCGCCCTGCTCGTCGCG-3'<br>5'-CGCGACGAGCAGGGCGCCGGATG-3' | Site-directed mutagenesis |
| | Tyr98His | Hist1H4E_Y98H_F<br>Hist1H4E_Y98H_R | 5'-GACAGGGACGCACTCTTCACGGCTTCGGC-3'<br>5'-GCCGAAGCCGTGAAGAGTGCGTCCCTGTC-3' | Site-directed mutagenesis |
| H4C6 (HIST1H4F) | WT | Hist1H4F_FL_F<br>Hist1H4F_FL_R | 5'-GCCACCATGTCTGGTAGAGGCAAAGGTG-3'<br>5'-TCAGCCACCAAAGCCGTACAG-3' | cDNA amplification |
| | Gly94Arg | Hist1H4F_G94R_F<br>Hist1H4F_G94R_R | 5'-CGCTCAAGCGCCAGAGACGCACTCTGTAC-3'<br>5'-GTACAGAGTGCGTCTCTGGCGCTTGAGCG-3' | Site-directed mutagenesis |
| H4C9 (HIST1H4I) | WT | Hist1H4I_FL_F<br>Hist1H4I_FL_R | 5'-GCCACCATGTCAGGACGCGGCAAAGGA-3'<br>5'-TTAGCCGCCGAAGCCATAGAG-3' | cDNA amplification |
| | His75Arg | Hist1H4I_H75R_F<br>Hist1H4I_H75R_R | 5'-ACCTACACGGAGCGCGCCAAGCGCAAG-3'<br>5'-CTTGCGCTTGGCGCGCTCCGTGTAGGT-3' | Site-directed mutagenesis |
| H4C11 (HIST1H4J) | WT | Hist1H4J_FL_F<br>Hist1H4J_FL_R | 5'-GCCACCATGTCTGGCCGCGGCAAAGGC-3'<br>5'-TAGGGTGGCCCTGAAAAGGGCC-3' | cDNA amplification |
| | Arg40Cys | Hist1H4J_R40C_F<br>Hist1H4J_R40C_R | 5'-GCCTTGCTCGCTGCGGCGGCGTG-3'<br>5'-CACGCCGCCGCAGCGAGCAAGGC-3' | Site-directed mutagenesis |

Table S3: Source data from the zebrafish RNA injection experiments at 50 pg, used to generate Fig 3D.

| | No injection | H4C3 WT | H4C3 P32A | H4C3 K91Q | H4C4 WT | H4C4 R40H | H4C5 WT | H4C5 K31T | H4C5 P32R | H4C5 R36W | H4C5 L37P | H4C5 R40C | H4C5 R45C | H4C5 Y98H | H4C6 WT | H4C6 G94R | H4C9 WT | H4C9 R40L | H4C9 H75R | H4C11 WT | H4C11 R40C |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Class I | 273 | 123 | 127 | 12 | 66 | 108 | 234 | 159 | 109 | 164 | 111 | 91 | 98 | 96 | 148 | 147 | 150 | 48 | 53 | 62 | 112 |
| Class II | 1 | 14 | 22 | 13 | 2 | 19 | 6 | 14 | 24 | 46 | 64 | 1 | 6 | 36 | 8 | 12 | 8 | 15 | 50 | 2 | 5 |
| Class III | 0 | 0 | 0 | 13 | 1 | 33 | 0 | 5 | 1 | 1 | 9 | 3 | 0 | 4 | 0 | 9 | 1 | 5 | 17 | 0 | 1 |
| Class IV | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 13 | 0 | 2 | 6 | 0 | 0 |
| Class V | 5 | 0 | 6 | 0 | 0 | 0 | 0 | 3 | 1 | 2 | 0 | 0 | 0 | 3 | 1 | 5 | 0 | 0 | 0 | 0 | 0 |
| Total | 279 | 137 | 155 | 50 | 69 | 160 | 240 | 183 | 135 | 213 | 184 | 95 | 106 | 139 | 157 | 186 | 159 | 70 | 126 | 64 | 118 |

Table S4: Source data from the zebrafish RNA injection experiments at 100 pg, used to generate Fig 3E.

| | No injection | H4C4 WT | H4C4 R40H | H4C5 WT | H4C5 R40C | H4C5 R45C | H4C11 WT | H4C11 R40C |
|---|---|---|---|---|---|---|---|---|
| Class I | 273 | 44 | 82 | 110 | 92 | 49 | 36 | 50 |
| Class II | 1 | 2 | 45 | 14 | 20 | 30 | 2 | 8 |
| Class III | 0 | 2 | 27 | 6 | 9 | 16 | 1 | 3 |
| Class IV | 0 | 0 | 2 | 0 | 2 | 17 | 0 | 2 |
| Class V | 5 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total | 279 | 49 | 156 | 130 | 123 | 112 | 39 | 63 |

## Supplemental Methods

### Patient Recruitment

Exome sequencing was undertaken for either research or clinical genetic testing, using standard pipelines at each referring centre. *De novo* status was confirmed by either trio-based exome sequencing and/or Sanger sequencing. Growth measurements were converted to Z-scores using the LSMgrowth method[1] or the Fenton 2013 growth chart[2] for preterm births.

### Protein Modelling

Variants were visualised on the human nucleosome structure (PDB code 5y0c[3]) using UCSF Chimera[4].

### Fish lines and husbandry

Zebrafish (*Danio rerio*) of the Tübingen longfin strain were kept in standard laboratory conditions[5]. Animal experiments were approved by the Animal Experimentation Committee of the Royal Netherlands Academy of Arts and Sciences.

### Expression assay in zebrafish embryos

Capped mRNA microinjections were carried out essentially as described[6]. Template human cDNA (H4C3, H4C4, H4C5, H4C6, H4C9 and H4C11) was used to generate novel cDNA encoding respectively H4C3 Pro32Ala, H4C4 Arg40His, H4C5 Leu37Pro, H4C5 Tyr98His, H4C5 Pro32Arg, H4C5 Arg35Trp, H4C6 Gly94Arg, H4C9 His75Arg and H4C11 Arg40Cys using oligonucleotides listed in Table S2. After cloning into pCS2GW by Gateway cloning (Life Technologies), the resulting template constructs were linearized with NotI-HF (New England BioLabs) and used for *in vitro* synthesis of capped mRNA with MESSAGE mMACHINE SP6 Ultra kit (Life Technologies). Microinjections in 1-cell-stage embryos were carried out with 50 pg or 100 pg of mRNA per embryo. After microinjection embryos were kept at 28.5 °C in E3 medium and development was assessed at approximately 28 hours post-fertilization. Phenotypical assessment data was collected for each histone variant over a minimum of two independent microinjection rounds.

## Imaging

Live observation of 28 hpf zebrafish embryos was carried out on a Zeiss StemiSV6 stereomicroscope (Carl Zeiss AG, Oberkochen, Germany). Image capture was performed with a Leica DFC420C digital microscope camera (Leica Microsystems, Wetzlar, Germany) mounted on a Zeiss Axioplan brightfield microscope (Carl Zeiss AG).

## Statistics

Statistics analysis was carried out with Prism 9 (Graphpad). Fisher's exact test was carried out on each histone H4 variant and the corresponding wild type. To carry out Fisher's test, we classified the scored phenotypes in only two outcomes: "no phenotype" (Class I) and "presence of a phenotype" (Class II + Class III + Class IV + Class V). The *P* values, levels of significance and number of embryos analyzed are reported in the figure and the figure legend. The source data used to generate figures 3D and E are shown in Tables S3 and S4, respectively.

## Supplemental References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