Molecular dissection of CHARGE syndrome highlights the vulnerability of neural crest cells to problems with alternative splicing and other transcription-related processes

Félix-Antoine Bérubé-Simard[1,2] and Nicolas Pilon[1,2,3]*

[1] Laboratoire de génétique moléculaire du développement, Département des sciences biologiques, Université du Québec à Montréal (UQAM), Montréal, QC, Canada.

[2] Centre d'Excellence en Recherche sur les Maladies Orphelines – Fondation Courtois (CERMO-FC), Université du Québec à Montréal (UQAM), Montréal, QC, Canada.

[3] Département de pédiatrie, Université de Montréal, Montréal, QC, Canada.

*** Correspondence to**: Nicolas Pilon (pilon.nicolas@uqam.ca)

KEYWORDS : Alternative splicing, CHARGE syndrome, Chromatin, Neural crest cells, Neurocristopathies, Spliceosomopathies.

ABSTRACT

CHARGE syndrome is characterized by co-occurrence of multiple malformations due to abnormal development of neural crest cells. Here, we review the phenotypic and molecular overlap between CHARGE syndrome and similar pathologies, and further discuss the observation that neural crest cells appear especially sensitive to malfunction of the chromatin-transcription-splicing molecular hub.

Introduction

Spliceosomopathies form a particular class of rare diseases characterized by the presence of mutations in spliceosome-associated factors [1, 2]. These congenital disorders present a complex array of phenotypes, including: microcephaly, intellectual disability, neurodegenerative conditions, limb malformations, congenital heart defects, and craniofacial malformations [3, 4, 5, 6]. Surprisingly, despite the fact that genes coding for spliceosome components are generally ubiquitously expressed, most of the anomalies described above affect neural tube and neural crest cell (NCC) derivatives.

Initially residing in the dorsal neural tube, NCCs are multipotent cells that colonize various regions of the developing embryo in order to generate very distinct cell types, such as: peripheral neurons and glia, hypothalamic GnRH neurons, craniofacial chondrocytes and osteoblasts, melanocytes, adrenal chromaffin cells as well as particular cell types of the heart, eye and inner ear [7]. The differentiation potential of NCCs is in part dictated by their original position along the anterior-posterior neural axis, generating 5 subpopulations of NCCs: cranial, cardiac, vagal, trunk and sacral. Problems with NCC migration, proliferation, survival, and/or differentiation may cause neurocristopathies, a heterogeneous group of pathologies that reflect the different NCC subpopulations affected as well as the wide diversity of potential NCC derivatives [8, 9]. For example, craniofacial malformations are caused by problems with cranial NCCs, while problems with cardiac and vagal NCCs may cause cardiac outflow tract and enteric nervous system malformations, respectively.

CHARGE syndrome is a neurocristopathy with complex clinical presentation that mainly impacts cranial and cardiac NCC derivatives, affecting ~1/10 000 newborns worldwide [10]. Although the acronym CHARGE stands for six of the anomalies that are commonly associated with the disorder (Coloboma of the eye, Heart defects, Atresia of choanae, Retardation of growth/development, Genital abnormalities, and Ear anomalies), diagnosis of CHARGE syndrome does not depend on the co-occurrence of all of them. Each of these characteristics as well as other anomalies frequently encountered (e.g. craniofacial dysmorphism and cleft palate) can vary from severe to absent, resulting in different combinations in different children [11]. Until very recently, heterozygous mutation of *CHD7* was the only known genetic cause of CHARGE syndrome [12]. Since CHD7 preferentially regulates nucleosome positioning near enhancer regions [13, 14], the only molecular mechanism suspected to be involved in CHARGE syndrome was chromatin remodeling-dependent transcriptional dysregulation of genes important for NCC development [15, 16, 17].

In a sustained and concerted effort to identify neurocristopathy-associated genes [18, 19, 20, 21], we recently generated a mouse model for *CHD7* mutation-negative CHARGE syndrome, by random insertional mutagenesis of the poorly characterized gene *Fam172a* [22]. We found that *Fam172a* codes for a new Ago2-binding protein that seems to be essential for stabilizing protein-protein interactions at the chromatin-spliceosome interface. Analysis of single and double *Fam172a-Chd7* mutant mice, as well as patient-derived cells, further allowed us to propose that dysregulation of cotranscriptional alternative splicing is a potentially unifying pathogenic mechanism for all cases of CHARGE syndrome, regardless of the mutated gene. In this point-of-view article, we now discuss our data in light of the recent identification of supplemental CHARGE syndrome-associated genes, and we also speculate as to why NCCs appear especially vulnerable to perturbation of chromatin structure-dependent cotranscriptional alternative splicing.

Fam172a: a new intriguing regulator of cotranscriptional alternative splicing

In the *Toupee* mouse model of CHARGE syndrome [22], mutagenic transgene sequences are inserted in the last intron of *Fam172a*, generating a hypomorphic allele that negatively affects almost every aspect of NCC ontology (migration, proliferation, survival and differentiation). Yet, we found that *Fam172a* expression is not restricted to this cell lineage, being instead almost ubiquitously expressed in embryonic day (e)8.5 to e12.5 embryos. Bioinformatics-based analysis of Fam172a protein sequences predicted different domains, including notably a large one originally described in *Saccharomyces pombe* Arb2 (Argonaute binding protein-2) [23]. Functionality of this domain was verified via immunofluorescence and co-immunoprecipitation assays, which showed that the Fam172a protein is mainly localized in the nucleus where it physically interacts with Ago2, but not Ago1. In accordance with the nuclear specificity of this interaction, a luciferase-based assay showed that decreased *Fam172a* expression has no impact on cytoplasmic posttranscriptional gene silencing. In addition, DNA and RNA were each found to be required for stabilizing the Fam172a-Ago2 interaction, suggesting these two proteins are part of a molecular process in which both types of nucleic acid are involved simultaneously, like alternative splicing.

A role for Fam172a in the regulation of cotranscriptional alternative splicing was then strengthened by transcriptomics and proteomics data from NCCs, which revealed massive dysregulation of gene expression levels and alternative splicing upon decreased expression of *Fam172a*, and enrichment for chromatin- and spliceosome-associated proteins in the Fam172a interactome. Among dysregulated genes, dozens previously described as being involved in the NCC gene regulatory network were notably identified, covering virtually every aspect of NCC development from

induction to terminal differentiation. Interestingly, we also found that Chd7 physically interacts with both Fam172a and Ago2, and that exogenous Fam172a can stimulate the Chd7-Ago2 interaction. Moreover, ChIP and RNA-ChIP assays demonstrated that both Fam172a and Chd7 are present on previously described Ago2-regulated alternatively spliced exons of *Cd44*. Using a larger set of previously described splicing targets of Ago2 [24], we then confirmed that alternative splicing is also extensively dysregulated in *Chd7* mutant mice as well as in cells from human patients bearing a mutation in *FAM172A*, *CHD7* or other unidentified genes. Importantly, acute treatments of human cells and mouse embryos with rapamycin – which downregulate ribosomal protein gene expression and thereby promote splicing of other pre-mRNAs by relieving competition for a limiting pool of splicing factors [25] – not only revealed that CHARGE syndrome-associated splicing defects can be corrected with a small molecule but also that this is sufficient to significantly decrease the severity of a highly penetrant CHARGE syndrome-associated phenotype (coloboma). This very comprehensive study thus allowed us to propose that dysregulation of cotranscriptional alternative splicing could well be the general pathogenic mechanism for CHARGE syndrome.

Dysfunction of the chromatin-transcription-splicing triumverate in CHARGE syndrome

Alternative splicing is now well known to be regulated by transcription and chromatin structure/composition – both of them influencing exon retention/exclusion by impacting RNA polymerase II elongation speed and/or splicing factor recruitment [26, 27] – and increasing evidence especially points to key roles for AGO [24, 28, 29, 30] and CHD proteins [31, 32, 33, 34]. While we were characterizing Fam172a's function, it was notably reported by another group that human CHD7can interact with the spliceosome and regulate alternative splicing [35]. In total agreement with our own findings, this work revealed that the spliceosome interactome (using the U2 snRNP as bait) not only contains CHD7 but also many other CHD and non-CHD chromatin remodelling factors, histones and regulators of histone post-translational modifications, transcriptional regulators, and components of the RNA interference pathway like AGO2. Furthermore, a central role for Chd7 in the regulation of alternative splicing is supported by ChIP-seq data from mouse ES cells [14], which showed that Chd7 occupies gene bodies almost to the same extent as for intergenic regions (36.6% *vs* 46%, respectively). As described in Figure 1, combining all these results to ours allowed us to propose a model in which Fam172a appears to act as a scaffolding protein at the chromatin-spliceosome interface [22].

Of note, the recent discovery of new CHARGE syndrome candidate genes [36, 37] also strongly supports the idea that dysregulation of chromatin structure-dependent cotranscriptional alternative splicing plays a fundamental role in CHARGE syndrome pathogenesis. Indeed, direct and/or indirect evidence for a regulatory role in alternative splicing exists for all new five candidate genes (*PUF60*, *EP300*, *RERE*, *KMT2D* and *KDM6A*): *PUF60* encodes a *bona fide* splicing factor [38, 39]; *EP300* codes for the p300 histone acetyltransferase, which has been shown to be directly involved in splicing regulation [40]; *RERE* codes for a transcriptional co-repressor that notably associates with HDAC1/2 [41, 42, 43], which are included in the Fam172a/spliceosome interactomes [22, 35]; *KMT2D* encodes a H3K4methyltransferase that is included in the spliceosome interactome [35], and trimethylation of H3K4 is known to trigger the recruitment of splicing factors [33]; and finally, *KDM6A* codes for a demethylase of H3K27me3, a histone mark associated with AGO-mediated splicing regulation [28]. Considering all of the above, we thus believe that a link with cotranscriptional alternative splicing would help prioritizing potentially damaging variants in *CHD7* mutation-negative individuals suspected to have CHARGE syndrome or a related pathology.

Extensive phenotypic overlap between neurocristopathies and spliceosomopathies highlights the vulnerability of neural crest cells to global splicing defects

Wide phenotypic variability (both inter- and intra-familial) is a well-known hallmark of CHARGE syndrome, explaining why there is also extensive clinical overlap between CHARGE syndrome (especially less severe cases) and many other developmental disorders caused by problems with cranial and cardiac NCCs (Table 1). These overlapping conditions notably include Kallmann (OMIM #308700, 147950), Kabuki (OMIM #147920, 300867) and 22q11.2 (OMIM #188400) syndromes as well as many craniofacial disorders known as spliceosomopathies, like Nager syndrome (OMIM #154400) and the Guion-Almeida type of mandibulofacial dysostosis (OMIM #610536). As the list of genes associated with these pathologies is extending, it is becoming clear that the observed clinical overlap can be explained by similarities at the genetic/molecular level (Table 1). In many cases, the same gene has even been associated with different clinical entities. For example, pathogenic variants in *CHD7* (typically associated with CHARGE syndrome) have been associated with Kallmann syndrome [44, 45] while pathogenic variants in both *KMT2D* and *KDM6A* (typically associated with Kabuki syndrome) have been associated with CHARGE syndrome [37]. Similarly, mutations of the spliceosome-associated protein encoding gene *EFTUD2*

(typically associated with the Guion-Almeida type of mandibulofacial dysostosis) have been found in individuals considered to have either CHARGE [46] or Nager [47] syndrome. In other words, all of the above neurocristopathy-related disorders are much more similar than originally thought, and all appear to be linked by defective regulation of cotranscriptional alternative splicing.

The tight interplay between chromatin structure/compostion, transcription and alternative splicing is critically important for virtually every single cell type, raising the question as to why neurocristopathies appear especially overrepresented upon perturbation of this large molecular hub. A potential reason could be that NCCs, like some other neural tube-derived cell types (motor neurons and retinal neurons), are simply more sensitive than other cell types to such perturbation. In strong support of this hypothesis, NCCs are already known to be particularly sensitive to a wide diversity of cellular stresses like hyperthermia [48], alcohol [49], zika virus [50], high glucose [51], oxidative stress [52] and nucleolar stress [53]. Many of these stresses lead to p53-mediated apoptosis, a process for which NCCs have also been shown to be sensitized to, at least in part because NCCs expresses higher levels of p53 compared to other cell types [54]. In this regard, it is interesting to note that p53 can be activated by splicing impairment [55] and that constitutively activated p53 results in a CHARGE-like phenotype in mouse embryos [56]. Yet, our work and work from others suggest that p53 activation could only be involved in a subset of *CHD7* mutation-positive cases [22, 56, 57].

The specific vulnerability of NCCs to dysregulated alternative splicing might also be explained by the particular importance of this molecular process in cell differentiation [58]. Indeed, as multipotent NCCs generate numerous and very distinct cell types over a relatively short period of time, it is conceivable that alternative splicing need to be tightly regulated in order to efficiently support the large number of cell fate switching events required along the way. In fact, we cannot think about another mechanism that would allow to quickly modify the proteome as needed for these cell diversification events to occur in time. This idea is notably supported by a recent analysis of cell diversification in the CNS [59], which revealed an association between particular switch exons in different genes and neuronal maturation stages.

Concluding remark

CHARGE syndrome is related to a large number of other rare genetic syndromes affecting cranial and cardiac NCCs, not only phenotypically but also molecularly speaking. Indeed, all of these conditions appear to potentially involve problems with the regulation of cotranscriptional

alternative splicing, to which NCCs seem especially vulnerable. Such extensive overlap suggests that many of these conditions are in fact variations of the same pathology, a possibility that might have direct clinical relevance for both diagnosis and therapy. On the one hand, this might facilitate genetic testing through targeted sequencing of a common set of candidate genes. On the other hand, this might open up the very interesting possibility of developing a universal *in utero* molecular therapy, regardless of the mutated gene.

FUNDING DETAILS

N.P. is a Senior Research Scholar of the Fonds de la recherche du Québec – Santé (FRQS) as well as the recipient of the UQAM Research Chair on Rare Genetic Diseases. The Pilon laboratory is funded by grants from the Canadian Institute of Health Research (CIHR), the Natural Science and Engineering Research Council of Canada (NSERC), the CHARGE syndrome Foundation and the Fondation du grand défi Pierre Lavoie. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

DISCLOSURE STATEMENT

No potential conflicts of interest were disclosed.

Figure 1. Potential mode of action of Fam172a in Ago2-mediated alternative splicing. Although the relationship between all players shown remains to be characterized in detail, current data suggest that the presence of Fam172a at the chromatin-spliceosome interface helps stabilizing protein-protein interactions between the small-RNA binding protein Ago2, chromatin remodelers such as Chd7 and histone modifiers such as Ehmt2. Model adapted from Refs. **[22, 24]**

REFERENCES

1. Lehalle D, Wieczorek D, Zechi-Ceide RM, et al. A review of craniofacial disorders caused by spliceosomal defects. Clin Genet. 2015 Nov;88(5):405-15. doi: 10.1111/cge.12596. PubMed PMID: 25865758.
2. Scotti MM, Swanson MS. RNA mis-splicing in disease. Nat Rev Genet. 2016 Jan;17(1):19-32. doi: 10.1038/nrg.2015.3. PubMed PMID: 26593421.
3. Czeschik JC, Voigt C, Alanay Y, et al. Clinical and mutation data in 12 patients with the clinical diagnosis of Nager syndrome. Hum Genet. 2013 Aug;132(8):885-98. doi: 10.1007/s00439-013-1295-2. PubMed PMID: 23568615.
4. Rudnik-Schoneborn S, Heller R, Berg C, et al. Congenital heart disease is a feature of severe infantile spinal muscular atrophy. J Med Genet. 2008 Oct;45(10):635-8. doi: 10.1136/jmg.2008.057950. PubMed PMID: 18662980.
5. Tsuiji H, Iguchi Y, Furuya A, et al. Spliceosome integrity is defective in the motor neuron diseases ALS and SMA. EMBO molecular medicine. 2013 Feb;5(2):221-34. doi: 10.1002/emmm.201202303. PubMed PMID: 23255347; PubMed Central PMCID: PMCPMC3569639.
6. Wieczorek D. Human facial dysostoses. Clin Genet. 2013 Jun;83(6):499-510. doi: 10.1111/cge.12123. PubMed PMID: 23565775.
7. Bronner ME, LeDouarin NM. Development and evolution of the neural crest: an overview. Dev Biol. 2012 Jun 1;366(1):2-9. doi: 10.1016/j.ydbio.2011.12.042. PubMed PMID: 22230617; PubMed Central PMCID: PMC3351559.
8. Bolande RP. Neurocristopathy: its growth and development in 20 years. Pediatr Pathol Lab Med. 1997 Jan-Feb;17(1):1-25. PubMed PMID: 9050057.
9. Etchevers HC, Amiel J, Lyonnet S. Molecular bases of human neurocristopathies. Adv Exp Med Biol. 2006;589:213-34. doi: 10.1007/978-0-387-46954-6_14. PubMed PMID: 17076285.
10. Hsu P, Ma A, Wilson M, et al. CHARGE syndrome: a review. Journal of paediatrics and child health. 2014 Jul;50(7):504-11. doi: 10.1111/jpc.12497. PubMed PMID: 24548020.
11. Hale CL, Niederriter AN, Green GE, et al. Atypical phenotypes associated with pathogenic CHD7 variants and a proposal for broadening CHARGE syndrome clinical diagnostic criteria. Am J Med Genet A. 2016 Feb;170(2):344-54. doi: 10.1002/ajmg.a.37435. PubMed PMID: 26590800.
12. Vissers LE, van Ravenswaaij CM, Admiraal R, et al. Mutations in a new member of the chromodomain gene family cause CHARGE syndrome. Nat Genet. 2004 Sep;36(9):955-7. doi: 10.1038/ng1407. PubMed PMID: 15300250.
13. Bouazoune K, Kingston RE. Chromatin remodeling by the CHD7 protein is impaired by mutations that cause human developmental disorders. Proc Natl Acad Sci U S A. 2012 Nov 20;109(47):19238-43. doi: 10.1073/pnas.1213825109. PubMed PMID: 23134727; PubMed Central PMCID: PMC3511097.
14. Schnetz MP, Handoko L, Akhtar-Zaidi B, et al. CHD7 targets active gene enhancer elements to modulate ES cell-specific gene expression. PLoS Genet. 2010 Jul;6(7):e1001023. doi: 10.1371/journal.pgen.1001023. PubMed PMID: 20657823; PubMed Central PMCID: PMC2904778.
15. Bajpai R, Chen DA, Rada-Iglesias A, et al. CHD7 cooperates with PBAF to control multipotent neural crest formation. Nature. 2010 Feb 18;463(7283):958-62. doi: 10.1038/nature08733. PubMed PMID: 20130577; PubMed Central PMCID: PMC2890258.
16. Fujita K, Ogawa R, Ito K. CHD7, Oct3/4, Sox2, and Nanog control FoxD3 expression during mouse neural crest-derived stem cell formation. The FEBS journal. 2016 Oct;283(20):3791-3806. doi: 10.1111/febs.13843. PubMed PMID: 27579714.

17. Schulz Y, Wehner P, Opitz L, et al. CHD7, the gene mutated in CHARGE syndrome, regulates genes involved in neural crest cell guidance. Hum Genet. 2014 Aug;133(8):997-1009. doi: 10.1007/s00439-014-1444-2. PubMed PMID: 24728844.
18. Bergeron KF, Cardinal T, Toure AM, et al. Male-Biased Aganglionic Megacolon in the TashT Mouse Line Due to Perturbation of Silencer Elements in a Large Gene Desert of Chromosome 10. PLoS Genet. 2015 Mar;11(3):e1005093. doi: 10.1371/journal.pgen.1005093. PubMed PMID: 25786024.
19. Bergeron KF, Nguyen CM, Cardinal T, et al. Upregulation of the Nr2f1-A830082K12Rik gene pair in murine neural crest cells results in a complex phenotype reminiscent of waardenburg syndrome type 4. Disease models & mechanisms. 2016 Nov 2;9(11):1283-1293. doi: 10.1242/dmm.026773. PubMed PMID: 27585883.
20. Pilon N. Pigmentation-based insertional mutagenesis is a simple and potent screening approach for identifying neurocristopathy-associated genes in mice. Rare Diseases. 2016 2016;4(1):e1156287.
21. Soret R, Mennetrey M, Bergeron KF, et al. A collagen VI-dependent pathogenic mechanism for Hirschsprung's disease. J Clin Invest. 2015 Dec 1;125(12):4483-96. doi: 10.1172/JCI83178. PubMed PMID: 26571399.
22. Belanger C, Berube-Simard FA, Leduc E, et al. Dysregulation of cotranscriptional alternative splicing underlies CHARGE syndrome. Proc Natl Acad Sci U S A. 2018 Jan 23;115(4):E620-E629. doi: 10.1073/pnas.1715378115. PubMed PMID: 29311329; PubMed Central PMCID: PMC5789929.
23. Buker SM, Iida T, Buhler M, et al. Two different Argonaute complexes are required for siRNA generation and heterochromatin assembly in fission yeast. Nat Struct Mol Biol. 2007 Mar;14(3):200-7. doi: 10.1038/nsmb1211. PubMed PMID: 17310250.
24. Ameyar-Zazoua M, Rachez C, Souidi M, et al. Argonaute proteins couple chromatin silencing to alternative splicing. Nat Struct Mol Biol. 2012 Oct;19(10):998-1004. doi: 10.1038/nsmb.2373. PubMed PMID: 22961379.
25. Munding EM, Shiue L, Katzman S, et al. Competition between pre-mRNAs for the splicing machinery drives global regulation of splicing. Mol Cell. 2013 Aug 08;51(3):338-48. doi: 10.1016/j.molcel.2013.06.012. PubMed PMID: 23891561; PubMed Central PMCID: PMC3771316.
26. Luco RF, Allo M, Schor IE, et al. Epigenetics in alternative pre-mRNA splicing. Cell. 2011 Jan 7;144(1):16-26. doi: 10.1016/j.cell.2010.11.056. PubMed PMID: 21215366; PubMed Central PMCID: PMC3038581.
27. Naftelberg S, Schor IE, Ast G, et al. Regulation of alternative splicing through coupling with transcription and chromatin structure. Annu Rev Biochem. 2015;84:165-98. doi: 10.1146/annurev-biochem-060614-034242. PubMed PMID: 26034889.
28. Allo M, Buggiano V, Fededa JP, et al. Control of alternative splicing through siRNA-mediated transcriptional gene silencing. Nat Struct Mol Biol. 2009 Jul;16(7):717-24. doi: 10.1038/nsmb.1620. PubMed PMID: 19543290.
29. Kalantari R, Chiang CM, Corey DR. Regulation of mammalian transcription and splicing by Nuclear RNAi. Nucleic Acids Res. 2016 Jan 29;44(2):524-37. doi: 10.1093/nar/gkv1305. PubMed PMID: 26612865; PubMed Central PMCID: PMC4737150.
30. Saint-Andre V, Batsche E, Rachez C, et al. Histone H3 lysine 9 trimethylation and HP1gamma favor inclusion of alternative exons. Nat Struct Mol Biol. 2011 Mar;18(3):337-44. doi: 10.1038/nsmb.1995. PubMed PMID: 21358630.
31. Gompers AL, Su-Feher L, Ellegood J, et al. Germline Chd8 haploinsufficiency alters brain development in mouse. Nat Neurosci. 2017 Aug;20(8):1062-1073. doi: 10.1038/nn.4592. PubMed PMID: 28671691; PubMed Central PMCID: PMCPMC6008102.

32. Murawska M, Brehm A. CHD chromatin remodelers and the transcription cycle. Transcription. 2011 Nov-Dec;2(6):244-53. doi: 10.4161/trns.2.6.17840. PubMed PMID: 22223048; PubMed Central PMCID: PMCPMC3265784.
33. Sims RJ, 3rd, Millhouse S, Chen CF, et al. Recognition of trimethylated histone H3 lysine 4 facilitates the recruitment of transcription postinitiation factors and pre-mRNA splicing. Mol Cell. 2007 Nov 30;28(4):665-76. doi: 10.1016/j.molcel.2007.11.010. PubMed PMID: 18042460; PubMed Central PMCID: PMC2276655.
34. Tai HH, Geisterfer M, Bell JC, et al. CHD1 associates with NCoR and histone deacetylase as well as with RNA splicing proteins. Biochem Biophys Res Commun. 2003 Aug 15;308(1):170-6. PubMed PMID: 12890497.
35. Allemand E, Myers MP, Garcia-Bernardo J, et al. A Broad Set of Chromatin Factors Influences Splicing. PLoS Genet. 2016 Sep;12(9):e1006318. doi: 10.1371/journal.pgen.1006318. PubMed PMID: 27662573; PubMed Central PMCID: PMC5035054.
36. Jordan VK, Fregeau B, Ge X, et al. Genotype-phenotype correlations in individuals with pathogenic RERE variants. Hum Mutat. 2018 May;39(5):666-675. doi: 10.1002/humu.23400. PubMed PMID: 29330883; PubMed Central PMCID: PMCPMC5903952.
37. Moccia A, Srivastava A, Skidmore JM, et al. Genetic analysis of CHARGE syndrome identifies overlapping molecular biology. Genet Med. 2018 Jan 4. doi: 10.1038/gim.2017.233. PubMed PMID: 29300383; PubMed Central PMCID: PMCPMC6034995.
38. Hastings ML, Allemand E, Duelli DM, et al. Control of pre-mRNA splicing by the general splicing factors PUF60 and U2AF(65). PLoS One. 2007 Jun 20;2(6):e538. doi: 10.1371/journal.pone.0000538. PubMed PMID: 17579712; PubMed Central PMCID: PMCPMC1888729.
39. Page-McCaw PS, Amonlirdviman K, Sharp PA. PUF60: a novel U2AF65-related splicing activity. Rna. 1999 Dec;5(12):1548-60. PubMed PMID: 10606266; PubMed Central PMCID: PMCPMC1369877.
40. Duskova E, Hnilicova J, Stanek D. CRE promoter sites modulate alternative splicing via p300-mediated histone acetylation. RNA biology. 2014;11(7):865-74. doi: 10.4161/rna.29441. PubMed PMID: 25019513; PubMed Central PMCID: PMCPMC4179961.
41. Plaster N, Sonntag C, Schilling TF, et al. REREa/Atrophin-2 interacts with histone deacetylase and Fgf8 signaling to regulate multiple processes of zebrafish development. Dev Dyn. 2007 Jul;236(7):1891-904. doi: 10.1002/dvdy.21196. PubMed PMID: 17576618.
42. Zhang S, Xu L, Lee J, et al. Drosophila atrophin homolog functions as a transcriptional corepressor in multiple developmental processes. Cell. 2002 Jan 11;108(1):45-56. PubMed PMID: 11792320.
43. Zoltewicz JS, Stewart NJ, Leung R, et al. Atrophin 2 recruits histone deacetylase and is required for the function of multiple signaling centers during mouse embryogenesis. Development. 2004 Jan;131(1):3-14. doi: 10.1242/dev.00908. PubMed PMID: 14645126.
44. Jongmans MC, van Ravenswaaij-Arts CM, Pitteloud N, et al. CHD7 mutations in patients initially diagnosed with Kallmann syndrome--the clinical overlap with CHARGE syndrome. Clin Genet. 2009 Jan;75(1):65-71. doi: 10.1111/j.1399-0004.2008.01107.x. PubMed PMID: 19021638; PubMed Central PMCID: PMC2854009.
45. Ogata T, Fujiwara I, Ogawa E, et al. Kallmann syndrome phenotype in a female patient with CHARGE syndrome and CHD7 mutation. Endocrine journal. 2006 Dec;53(6):741-3. PubMed PMID: 16960397.
46. Gordon CT, Petit F, Oufadem M, et al. EFTUD2 haploinsufficiency leads to syndromic oesophageal atresia. J Med Genet. 2012 Dec;49(12):737-46. doi: 10.1136/jmedgenet-2012-101173. PubMed PMID: 23188108.

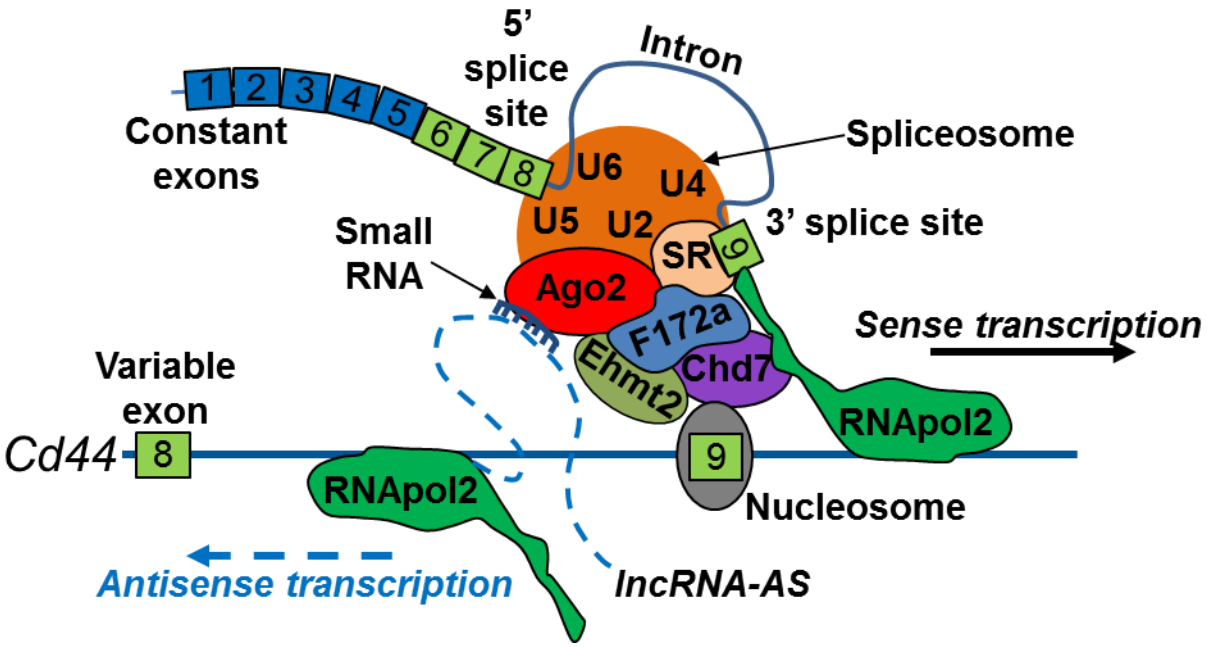
47. Bernier FP, Caluseriu O, Ng S, et al. Haploinsufficiency of SF3B4, a component of the pre-mRNA spliceosomal complex, causes Nager syndrome. Am J Hum Genet. 2012 May 04;90(5):925-33. doi: 10.1016/j.ajhg.2012.04.004. PubMed PMID: 22541558; PubMed Central PMCID: PMC3376638.
48. Hutson MR, Keyte AL, Hernandez-Morales M, et al. Temperature-activated ion channels in neural crest cells confer maternal fever-associated birth defects. Sci Signal. 2017 Oct 10;10(500). doi: 10.1126/scisignal.aal4055. PubMed PMID: 29018170.
49. Smith SM, Garic A, Flentke GR, et al. Neural crest development in fetal alcohol syndrome. Birth defects research Part C, Embryo today : reviews. 2014 Sep;102(3):210-20. doi: 10.1002/bdrc.21078. PubMed PMID: 25219761; PubMed Central PMCID: PMCPMC4827602.
50. Bayless NL, Greenberg RS, Swigut T, et al. Zika Virus Infection Induces Cranial Neural Crest Cells to Produce Cytokines at Levels Detrimental for Neurogenesis. Cell Host Microbe. 2016 Oct 12;20(4):423-428. doi: 10.1016/j.chom.2016.09.006. PubMed PMID: 27693308; PubMed Central PMCID: PMCPMC5113290.
51. Wang XY, Li S, Wang G, et al. High glucose environment inhibits cranial neural crest survival by activating excessive autophagy in the chick embryo. Scientific reports. 2015 Dec 16;5:18321. doi: 10.1038/srep18321. PubMed PMID: 26671447; PubMed Central PMCID: PMC4680872.
52. Morgan SC, Relaix F, Sandell LL, et al. Oxidative stress during diabetic pregnancy disrupts cardiac neural crest migration and causes outflow tract defects. Birth Defects Res A Clin Mol Teratol. 2008 Jun;82(6):453-63. doi: 10.1002/bdra.20457. PubMed PMID: 18435457; PubMed Central PMCID: PMCPMC5452612.
53. Jones NC, Lynn ML, Gaudenz K, et al. Prevention of the neurocristopathy Treacher Collins syndrome through inhibition of p53 function. Nat Med. 2008 Feb;14(2):125-33. doi: nm1725 [pii]

10.1038/nm1725. PubMed PMID: 18246078; PubMed Central PMCID: PMC3093709. eng.
54. Calo E, Gu B, Bowen ME, et al. Tissue-selective effects of nucleolar stress and rDNA damage in developmental disorders. Nature. 2018 Feb 1;554(7690):112-117. doi: 10.1038/nature25449. PubMed PMID: 29364875; PubMed Central PMCID: PMCPMC5927778.
55. Allende-Vega N, Dayal S, Agarwala U, et al. p53 is activated in response to disruption of the pre-mRNA splicing machinery. Oncogene. 2013 Jan 03;32(1):1-14. doi: 10.1038/onc.2012.38. PubMed PMID: 22349816.
56. Van Nostrand JL, Brady CA, Jung H, et al. Inappropriate p53 activation during development induces features of CHARGE syndrome. Nature. 2014 Oct 9;514(7521):228-32. doi: 10.1038/nature13585. PubMed PMID: 25119037; PubMed Central PMCID: PMC4192026.
57. Balow SA, Pierce LX, Zentner GE, et al. Knockdown of fbxl10/kdm2bb rescues chd7 morphant phenotype in a zebrafish model of CHARGE syndrome. Dev Biol. 2013 Oct 01;382(1):57-69. doi: 10.1016/j.ydbio.2013.07.026. PubMed PMID: 23920116; PubMed Central PMCID: PMC3816111.
58. Fiszbein A, Kornblihtt AR. Alternative splicing switches: Important players in cell differentiation. Bioessays. 2017 Jun;39(6). doi: 10.1002/bies.201600157. PubMed PMID: 28452057.
59. Weyn-Vanhentenryck SM, Feng H, Ustianenko D, et al. Precise temporal regulation of alternative splicing during neural development. Nature communications. 2018 Jun 6;9(1):2189. doi: 10.1038/s41467-018-04559-0. PubMed PMID: 29875359; PubMed Central PMCID: PMCPMC5989265.

Table 1. Clinical and molecular overlap between CHARGE syndrome and other rare conditions

Pathology	Clinical characteristics*	Causative Gene(s)
CHARGE syndrome (OMIM #214800)	- Brain (e.g. corpus callosum agenesis, olfactory bulb hypoplasia microcephaly) - Cardiac (e.g. septal defects, patent ductus arteriosus, Tetralogy of Fallot) - Cranial nerves (e.g. facial palsy, swallowing difficulties) - Craniofacial (e.g. square face, flat midface, facial asymmetry, downslanting palpebral fissures) - Ears (e.g. small and cup-shaped external ears, hypoplastic semi-circular canals) - Eyes (e.g. coloboma, microphthalmia) - Genitourinal (e.g. cryptorchidism, hypogonadotropic hypogonadism, uterus agenesis, kidney hypoplasia) - Growth retardation and intellectual disability - Immunological (e.g. thymus hypoplasia, immunodeficiency) - Oronasal (e.g. choanal atresia, cleft palate/lip) - Sensorineural (anosmia, hearing loss) - Skeletal (e.g. scoliosis, limb and hand anomalies) - Laryngopharyngeal (e.g. tracheoesophageal fistula, parathyroid hypoplasia)	Chromatin factors *CHD7*(major) , *EP300, KMT2D*, *KDM6A* (uncertain) Chromatin-spliceosome interface *FAM172A* Splicing factors *PUF60, EFTUD2* (uncertain) Transcription factors *RERE* Other *SEMA3E* (uncertain)
22q11.2 syndrome (OMIM #188400)	- Cardiac (e.g. septal defects, patent ductus arteriosus, Tetralogy of Fallot) - Craniofacial (e.g. telecanthus, short palbebral fissure, micrognathia, square nasal tip) - Ears (e.g. low-set and abnormally-folded external ear) - Growth retardation and intellectual disability - Immunological (e.g. thymus hypoplasia) - Oronasal (e.g. small mouth, cleft palate) - Laryngopharyngeal (parathyroid hypoplasia) - Sensorineural (hearing loss)	Transcription factors *TBX1*(major)
Acrofacial dystosis 1, Nager type (OMIM #154400)	- Craniofacial (micrognathia, malar hypoplasia, downslanting palpebral fissures, midface retrusion) - Ears (e.g. small and abnormally-folded external ear) - Eyes (e.g. eyelid coloboma) - Oronasal (e.g. cleft palate/lip) - Sensorineural (hearing loss) - Skeletal (e.g. limb and hand anomalies)	Splicing factors *SF3B4* (major), *EFTUD2* (uncertain)
Kabuki syndrome (OMIM #147920, 300867)	- Brain (e.g. microcephaly) - Cardiac (e.g. septal defects, patent ductus arteriosus, Tetralogy of Fallot) - Craniofacial (e.g. long palpebral fissures, arched eyebrows, broad and depressed nasal tip, dental agenesis) - Ears (e.g. large and abnormally-folded external ears) - Eyes (e.g. strabismus) - Genitourinal (e.g. early puberty) - Growth retardation and intellectual disability - Immunological (e.g. immune deficiency) - Oronasal (e.g. cleft palate) - Skeletal (e.g. scoliosis, limb and hand anomalies)	Chromatin factors *KMT2D* (major), *KDM6A*
Kallmann syndrome (OMIM #308700, 147950)	- Brain (e.g. olfactory bulb hypoplasia) - Craniofacial (e.g. dental agenesis) - Genitourinal (e.g. cryptorchidism, hypogonadotropic hypogonadism, kidney agenesis) - Growth retardation and intellectual disabillity - Oronasal (e.g. cleft palate/lip) - Sensorineural (anosmia, hearing loss)	Chromatin factors *CHD7* Other *FGFR1* (major) , *FGF8, KAL1, PROKR2, GNRH*
Mandibulofacial dystosis, Guion-Almeida type (OMIM #610536)	- Brain (e.g. microcephaly) - Cardiac (e.g. septal defects, patent ductus arteriosus) - Craniofacial (midface and malar hypoplasia, micrognathia, oblique palpebral fissure) - Ears (e.g. low-set and abnormally-folded external ears, preauricular tags, inner ear malformations, conductive hearing loss) - Laryngopharyngeal (e.g. tracheoesophageal fistula) - Growth retardation and intellectual disabillity - Oronasal (e.g. choanal atresia, cleft palate)	Splicing factors *EFTUD2*

Note: *all these characteristics do not usually co-occur in a given patient*


5'
splice
site
Intron
1
2
3
4
5
6
7
8
Constant
exons
U6
U4
U5
U2
Spliceosome
3' splice site
Small
RNA
SR
9
Ago2
F172a
Chd7
Ehmt2
Sense transcription
Variable
exon
Cd44
8
RNApol2
9
Nucleosome
RNApol2
Antisense transcription
lncRNA-AS