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Developmental processes regulate craniofacial variation in disease and evolution

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Abstract

Variation in development mediates phenotypic differences observed in evolution and disease. Although the mechanisms underlying phenotypic variation are still largely unknown, recent research suggests that variation in developmental processes may play a key role. Developmental processes mediate genotype-phenotype relationships and consequently play an important role regulating phenotypes. In this review, we provide an example of how shared and interacting developmental processes may explain convergence of phenotypes in spliceosomopathies and ribosomopathies. These data also suggest a shared pathway to disease treatment. We then discuss three major mechanisms that contribute to variation in developmental processes: genetic background (gene-gene interactions), gene-environment interactions, and developmental stochasticity. Finally, we comment on evolutionary alterations to developmental processes, and the evolution of disease buffering mechanisms.

Keywords

morphological variation; craniofacial anomalies; evolution of development; genotype-phenotype relationships; ribosomopathies; spliceosomopathies

Introduction:

Variation in development underlies phenotypic differences in evolution and disease. Despite the importance of developmental variation, mechanisms underlying its generation are still poorly understood. In the post-genomic area, significant research in developmental biology has been devoted to understanding gene function. In such studies, discussion of phenotypic variation is typically minimized in order to better reveal causal genotype-phenotype relationships. Similarly, candidate gene and genome-wide association studies have been commonly employed to correlate genetic information with disease phenotypes (Liu *et al.*, 2012; Yu *et al.*, 2017). However, as more of these studies are reported, it is becoming increasingly clear that genotype-phenotype relationships are complex, and that phenotypes associated with loss of function mutations can be quite variable (Hallgrimsson *et al.*, 2009; Parsons *et al.*, 2008). Recent research has explicitly focused on variation as key to understanding genotype-phenotype relationships. In particular, the role of development in

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mediating phenotypic variance has helped explain variation in phenotypic penetrance (Green *et al.*, 2017; Young *et al.*, 2014).

With very few exceptions, mutations affecting individual genes do not have a predictable phenotypic outcome. While some cellular diseases, such as sickle cell anemia, exhibit high penetrance, similar precise genotype-phenotype correlations are rarely observed in complex morphological structures. In fact, the presence of disease-causing mutations in healthy subjects indicates that incomplete penetrance of Mendelian disorders may be more common than expected (Chen *et al.*, 2016). This is especially true for craniofacial phenotypes, which involve particularly complex genotype-phenotype relationships (Hallgrimsson *et al.*, 2014). Craniofacial development involves many genes of small effect (Porto *et al.*, 2016), which work together in sophisticated protein complexes that regulate developmental processes, and facial phenotypes result from the summation of many hierarchical developmental processes (Fish, 2016; Hallgrimsson *et al.*, 2009; Porto *et al.*, 2016).

Identifying developmental processes as central mediators of genotype-phenotype relationships has several implications for disease diagnosis and treatment. First, mutations in genes that contribute to related developmental processes are predicted to share disease phenotypes. Second, identification of specific developmental processes disrupted by disease mutations may provide avenues for therapeutic treatment at the metabolic, rather than genetic, level. To provide an example of how developmental processes mediate genotype-phenotype relationships, we first review recent research on spliceosomopathies and ribosomopathies suggesting that the pathogenesis of these diseases may be related through interacting developmental processes upstream of protein synthesis. Facial development requires many such developmental processes, therefore, understanding why and how developmental processes vary is important to understanding variation in the penetrance and/or severity of disease mutations. We describe mechanisms contributing to variation in developmental processes, including gene-gene interactions, gene-environment interactions, and developmental stochasticity. Finally, we comment on evolutionary alterations to developmental processes, and the evolution of disease buffering mechanisms.

Developmental processes linking spliceosomopathies and ribosomopathies

Spliceosomopathies

Spliceosomopathies are caused by defects in the splicing machinery. Pre-mRNA splicing is the molecular process that ensures the ligation of exons encoded by a specific gene (Will and Luhrmann, 2011). This highly orchestrated process is mediated by a large ribonucleoprotein (RNP) complex called the spliceosome. A nascent mRNA molecule can contain constitutive as well as variable exons. The difference between these two types of exons lies in the frequency at which they are included in the mature mRNA. While constitutive exons are always a part of the mature mRNA, the inclusion of variable exons depends on different spatiotemporal contexts (Kriventseva *et al.*, 2003). Alternative splicing is the primary mechanism used by our cells to produce different versions (isoforms) of a specific mRNA molecule in order to expand the transcriptomic repertoire and increase proteomic diversity

(Nilsen and Graveley, 2010). The expression of certain, alternatively spliced, isoforms is important during tissue development (Baralle and Giudice, 2017). Therefore, the de-regulation of factors that promote splicing results in numerous human diseases.

Several human congenital disorders classified as mandibulofacial (MFD) or acrofacial dysostoses (AFD) are linked to mutations in genes coding for splicing factors. MFDs are a group of congenital diseases that arise from the abnormal development of the first and second pharyngeal arches whereas AFDs are a subdivision of MFDs that also involve limb defects (Wieczorek, 2013). Mutations in genes known to code for splicing factors such as *SF3B4*, *EFTUD2*, *TXNL4A*, *EIF4A3*, or *SNRPB* have recently been identified as disease-causing genes in individuals with MFDs or AFDs (Lehalle *et al.*, 2015). Mutations in *SF3B4* or *EFTUD2* are commonly found in patients diagnosed with Nager syndrome or Mandibulofacial Dysostosis Guion-Almeida type (MFDGA), respectively (Bernier *et al.*, 2012; Lines *et al.*, 2012).

Most spliceosomopathies share similar craniofacial phenotypes (Table 2). Nager syndrome is characterized by a facial phenotype that includes malar and mandibular hypoplasia, down-slanting palpebral fissures, external ear defects, and cleft palate (Bernier *et al.*, 2012; Czeschik *et al.*, 2013; Petit *et al.*, 2014). The most common limb defects associated with Nager syndrome, which contribute to its classification as an AFD, include pre-axial limb defects, primarily hypoplastic or absent thumbs (Bernier *et al.*, 2012; Czeschik *et al.*, 2013; Petit *et al.*, 2014). Patients diagnosed with MFDGA have a facial phenotype that overlaps with that of Nager syndrome but also involves microcephaly. Additionally, individuals with MFDGA occasionally exhibit pre-axial limb defects such as proximally placed thumbs or polydactyly of thumbs (Guion-Almeida *et al.*, 2006; Lines *et al.*, 2012).

SF3B4 codes for a splicing factor (known as SF3B4, SAP49, or SF3B49) which is one of the seven proteins that make up the SF3B complex of the U2 snRNP (Will and Luhrmann, 2011). SF3B4 binds upstream of the branch site in nascent mRNA molecules and interacts with other factors, especially SAP145, to tether the U2 snRNP to the branch site (Champion-Arnaud and Reed, 1994). In *Xenopus*, knock-down of *Sf3b4* phenocopies the craniofacial defects of human patients with Nager Syndrome (Devotta *et al.*, 2016). Functional studies in *Xenopus* showed that *Sf3b4* is required for the formation and survival of neural crest cells (NCCs) and causes facial defects due to a reduction in progenitors. Devotta and colleagues (2016) found that some genes involved in NCC development were down regulated, however, they did not find evidence to support the hypothesis that the down-regulation of these genes was associated with defective splicing. In contrast, a recent study by Marques and colleagues (2016) in human tissue showed that mRNA splicing is impaired in chondrocytes of fetuses with Rodriguez syndrome, which is a more severe and lethal form of Nager syndrome that is also caused by mutations in *SF3B4*. SF3B4 has also been reported to bind to BMPR-IA and inhibit BMP-mediated osteochondral cell differentiation, which may also contribute to skeletal defects (Nishanian and Waldman, 2004; Watanabe *et al.*, 2007).

MFDGA is associated with mutations in *EFTUD2*, which encodes the spliceosomal GTPase U5–116kD that is part of the U5 snRNP (Fabrizio *et al.*, 1997; Lines *et al.*, 2012). The function of EFTUD2 has been less well studied, however, the *S. cerevisiae* homolog of U5–

116kD, Snu114p, is involved in spliceosome activation by regulating the dissociation of the U4 and U6 RNAs (Bartels *et al.*, 2002). Further, EFTUD2 interacts with SF3B4 in the human spliceosome (Hegele *et al.*, 2012). Together, these data suggest that disease phenotypes associated with Nager Syndrome and MFDGA are caused by defective splicing.

Charge syndrome (Fam and Chd7)

CHARGE syndrome is a human congenital disorder that involves a combination of phenotypes emphasized in the acronym for CHARGE, which is coloboma of the eye, heart defects, atresia of choanae, retardation of growth and development, genital abnormalities, and ear anomalies (Pagon *et al.*, 1981). CHARGE syndrome is associated with mutations in *CHD7* (chromodomain helicase DNA-binding protein 7) which are thought to result in haploinsufficiency (Vissers *et al.*, 2004). CHD7 is known to regulate the expression of important genes in the NCC gene regulatory network and has been identified as one of the chromatin factors that might affect the outcome of splicing (Bajpai *et al.*, 2010; Schulz *et al.*, 2014). Although loss of function mutations in *CHD7* are associated with CHARGE, up to 30% of patients with the disease do not test positive for *CHD7* mutations (Zentner *et al.*, 2010).

A recent publication by Belanger and colleagues (2018) showed that the pathogenic mechanism that underlies CHARGE syndrome consists of the dysregulation of co-transcriptional alternative splicing. They used the *Toupee* mouse line as a model for investigating *CHD7* mutation-negative CHARGE. The *Toupee* line was generated by insertion of a *tyrosinase* minigene into the FVB/N genetic background in a locus that controls NCC development (Pilon, 2016). $Toupee^{Tg/Tg}$ NCCs migrate more slowly than those of wild-type mice, and they show a significant decrease in proliferation and an increase in apoptosis (Belanger *et al.*, 2018). The transgene insertion site of the *Toupee* line was determined to be the last intron of *Fam172a* which is a highly conserved gene between mouse and human. *Fam172a* is ubiquitously expressed during development of wild-type embryos and down-regulated in $Toupee^{Tg/Tg}$ embryos. Double-immunofluorescence and coimmunoprecipitation experiments revealed that Fam172a interacts with Argonaute 2 (Ago2) in the nucleus. Argonaute proteins, specifically AGO1 and AGO2, are involved in the regulation of alternative splicing by coupling chromatin structure to RNA polymerase (Pol) II elongation (Ameyar-Zazoua *et al.*, 2012). Further, they showed that among the binding partners of Fam172a there is an enrichment for chromatin proteins and splicing factors. Exogenous Fam172a was found to promote the interaction between Chd7 and Ago2. Furthermore, transcriptome analysis of $Toupee^{Tg/Tg}$ NCCs revealed that 30% of all aberrantly spliced transcripts correspond to genes that are also affected during transcription. Collectively, this data supports the hypothesis that Fam172a, Ago2, and Chd7 interact in a complex with chromatin to regulate alternative splicing (Belanger *et al.*, 2018).

The results of Bélager and colleagues (2018) are intriguing because they suggest that CHARGE syndrome may be a spliceosomopathy. To test their hypothesis, they treated lymphoblastic cell lines (LCLs) derived from patients with CHD7 mutation-negative CHARGE with rapamycin. Rapamycin is known to suppress the TOR pathway which, when unperturbed, promotes ribosome biogenesis by upregulating ribosomal gene expression (Li

et al., 2014; Martin *et al.*, 2004). Rapamycin treatment causes a decrease in the expression of ribosomal protein genes, which in turn results in an increase in the number of available splicing factors that can promote the splicing of other pre-mRNAs (Munding *et al.*, 2013). When LCLs from CHARGE patients were treated with rapamycin, the defective splicing of four genes expressed was rescued (Belanger *et al.*, 2018). Taken together, these results suggest that the dysregulation of co-transcriptional alternative splicing is the pathogenic mechanism that underlies both CHD7 mutation-negative and CHD7 mutation-positive cases of CHARGE (Belanger *et al.*, 2018). Further, these data suggest that alternative splicing and ribosome biogenesis are interacting developmental processes (Fig. 1).

Ribosomopathies

Ribosomopathies are caused by defects in ribosome biogenesis. Ribosomes are large RNP complexes composed of 4 ribosomal RNAs (rRNAs) and at least 80 RNA binding proteins that translate spliced mRNA into protein (Liu and Ellis, 2006). Ribosome biogenesis involves all three RNA polymerases and hundreds of other proteins involved in rRNA maturation and assembly into small and large subunits. This process is temporally and spatially separated, with Pol I-mediated transcription of rRNA in the nucleolus, and Pol II-mediated transcription of ribosomal protein genes in the nucleoplasm (Russo and Russo, 2017). Pol III synthesis of 5S rRNA also occurs in the nucleoplasm. Maturation of ribosomes is completed in the cytoplasm after nuclear export (Henras *et al.*, 2015). Thus, ribosome biogenesis consists of a hierarchal series of processes upstream of protein synthesis (Fig. 1).

A number of syndromes are characterized as ribosomopathies, most notably Treacher Collins Syndrome (TCS). TCS is characterized by midfacial hypoplasia, micrognathia with or without cleft palate, underdeveloped external ears and inner ear anomalies with hearing loss, coloboma, and downward slanting eyes (Terrazas *et al.*, 2017). TCS is caused predominantly by mutations in *TCOF1*, with mutations in *POLR1D* and *POLR1C* associated with some *TCOF1* mutation-negative cases (Terrazas *et al.*, 2017). TCOF1 is a nucleolar phosphoprotein implicated in Pol I transcription of rRNA (Larsen *et al.*, 2014). In mice, heterozygous mutations in *Tcof1* cause massive apoptosis of pre-migratory NCCs, leading to craniofacial hypoplasia that phenocopies TCS malformations in human patients (Dixon *et al.*, 2006). Craniofacial defects caused by *Tcof1* haploinsufficiency can be rescued by inhibition or down-regulation of p53, which reduces NCC apoptosis (Jones *et al.*, 2008).

POLR1C and POLR1D are subunits of RNA Pol I and III, which mediate rRNA transcription. Similar to *Tcof1* mutations in mice, *polr1c* and *polr1d* loss-of-function in zebrafish reduces ribosome biogenesis, causes NCC apoptosis, and generates defects of the facial skeleton suggestive of TCS. Additionally, the facial cartilage defects can mostly be rescued by inhibiting p53 (Noack Watt *et al.*, 2016). These data suggest a similar pathogenic mechanism for *TCOF1*, *POLR1D*, and *POLR1C*. Recent work suggests that a shared mechanism in TCS may be Pol I transcriptional stress, which causes loss of DDX21 from chromatin and its re-localization from the nucleolus to the nucleoplasm (Calo *et al.*, 2015). DDX21 is a DEAD-box RNA helicase involved in both nucleolar Pol I transcription of rRNA and subsequent Pol II-mediated transcription of ribosomal proteins in the

nucleoplasm (Calo *et al.*, 2015). Reduction of *POLR1D* or *TCOF1* causes DDX21 re-localization to the nucleoplasm, and preventing DDX21 loss from the nucleolus rescues craniofacial defects in *tcof1* deficient *Xenopus* embryos. Further, knock-down of DDX21 alone induces NCC apoptosis and TCS-like craniofacial defects (Calo et al., 2018).

As with spliceosomopathies, some ribosomopathies affect the craniofacial complex, while others have more widespread effects including axial and limb defects (Yelick and Trainor, 2015). Interestingly, DDX21 dysfunction is also observed upon knock-down of genes associated with other ribosomopathies, such as Diamond-Blackfan anemia (Calo *et al.*, 2018). Taken together, these data suggest that p53 activation and DDX21 re-localization downstream of rDNA damage caused by Pol I transcriptional stress may be a common mediator of ribosomopathies (Calo *et al.*, 2018).

Alternative splicing and ribosomal biogenesis interact upstream of protein synthesis

Studies in multiple model systems indicate that ribosomopathies share a pathogenic mechanism, which is p53 mediated cell death (Calo *et al.*, 2018; Mills and Green, 2017). Similarly, studies in *Xenopus*, zebrafish, and mouse models also reveal that haploinsufficiency of genes coding for splicing factors ultimately results in increased cell death (Belanger *et al.*, 2018; Devotta *et al.*, 2016; Lei *et al.*, 2016). Additionally, CHD7 deficiency causes an increase in p53 activation in mouse NCCs and human fibrobalsts (Van Nostrand *et al.*, 2014). CHD7 is also reported to negatively regulate p53 expression by binding its promoter which could be one of the mechanisms that explains how low levels of CHD7 result in an upregulation of p53 expression (Van Nostrand *et al.*, 2014). This suggests that NCC death could also be a pathogenic mechanism underlying CHARGE syndrome.

In a recent study, Zhang and colleagues describe how the ribosomal proteins Rpl22 and Rpl22-Like1 (Rpl22l1) perform extra-ribosomal functions by acting in the nucleoplasm to regulate the splicing of *Smad2* in *Xenopus* embryos (Zhang *et al.*, 2017). In this case ribosomal and splicing factors interact to control morphogenesis, further suggesting that these two molecular processes are linked.

The convergence on a similar cellular outcome, such as pre-migratory NCC death, might explain why ribosomopathies and spliceosomopaties have overlapping craniofacial phenotypes. But why should a deficit in a general regulator of cell function have cell-type-specific effects? NCC development involves multiple developmental processes, including induction, specification, epithelial-mesenchymal transition (EMT), delamination, and migration (Sauka-Spengler and Bronner-Fraser, 2008). EMT, in particular, involves significant changes in cell polarity and adhesion, which requires significant changes in gene expression, protein turnover, and metabolic inputs (Kalluri and Weinberg, 2009). It is at this stage that the survival and proliferation of NCCs appear to be particularly sensitive to perturbation, which may imply a high demand for protein synthesis in these cells. However, rescue of NCC survival by down-regulation of p53 in Tcof-deficient mice occurs independently of ribosome biogenesis (Jones *et al.*, 2008). This suggests that deficits in ribosome biogenesis can be compensated for in NCCs, as they are in other cell types.

Recently, Calo and colleagues (2018) addressed the specific question of why mutations affecting the ribosome biogenesis pathway particularly impact NCCs. They showed that NCCs are sensitized to p53 stabilization and are 2-fold more likely than other embryonic cell types to undergo apoptosis when treated with a p53 stabilizing drug. When impaired, molecular processes such as alternative splicing and ribosome biogenesis result in an increase of p53 expression and activation (Allende-Vega *et al.*, 2013; Dixon *et al.*, 2006). In normal development, NCC express high levels of p53 relative to other cells (Calo *et al.*, 2018; Rinon *et al.*, 2011). High levels of *p53* may be necessary to regulate NCC proliferation during EMT (Rinon *et al.*, 2011). Thus, the complex development of NCCs, involving high p53 expression, may explain their susceptibility to defects in general regulators of cell function.

Diseases caused by interacting developmental processes may share a metabolic treatment

Patients with AFDs or MFDs are diagnosed based on a series of phenotypes that overlap and can be subtly different (Green *et al.*, 2013). For example, Diamond-Blackfan anemia with Mandibulofacial Dysostosis, a ribosomopathy, involves phenotypes that are also seen in MFDGA, a spliceosomopathy (Gripp *et al.*, 2014). Often times, patients who are diagnosed with one syndrome are later found to harbor mutations linked to a different syndrome that is phenotypically similar to the first one (Bernier *et al.*, 2012; Gordon *et al.*, 2012; Vincent *et al.*, 2016). The absence of precise genotype-phenotype correlations may be due to the fact that the different mutations occur in genes functioning in the same and/or closely interacting developmental processes. Individual differences in genetic background, environmental influences, or developmental stochasticity, as described below, could also contribute to variability in phenotypes, thus further complicating genotype-phenotype correlations. However, this complication could be a benefit to disease treatment rather than an obstacle. Therapies that attempt to restore protein function due to loss-of-function mutations have achieved little success (Dietz, 2010). A promising alternative is to focus on modifiers that buffer or compensate for reductions in protein function (Chen *et al.*, 2016). Increased understanding of how genes relate to developmental processes will be an important step to facilitate this therapeutic alternative.

Variation in developmental processes

In the above discussion, we have described how many genes contribute to a single developmental process, and how multiple developmental processes contribute to a single cellular phenotype (NCC survival). This can explain how mutations in seemingly unrelated genes can cause broadly similar phenotypes. However, disease causing mutations also exhibit variation in penetrance and severity, which may also be explained by considering phenotypes from the perspective of developmental processes. Therefore, understanding why and how developmental processes vary is important to understanding phenotypic variation. Factors contributing to variation in developmental processes include gene-gene interactions, gene-environment interactions, and developmental stochasticity.

Gene-gene interactions

Developmental processes rely on the interaction of many different gene products. Therefore, phenotypic outcomes of mutations affecting a target gene may be modified by its genetic background, that is, the genotype of all the other genes it interacts with in the regulation of a particular process. Such interactions between a target gene and its modifiers are referred to as epistasis. Epistatic interactions can affect the dominance, penetrance, expressivity, and pleiotropy of a mutation (Mackay, 2014; Nadeau, 2001; Riordan and Nadeau, 2017). Dominance modifiers cause heterozygotes to develop disease phenotypes similar to homozygous mutants.

Penetrance modifiers affect the frequency, but not severity, of disease. For example, Pfeiffer syndrome is an autosomal dominant disorder caused by mutations in FGF receptors (FGFRs) that is characterized by craniosynostosis, and other dysmorphic facial features, including bulging eyes, a high forehead, mid-facial hypoplasia, and micrognathia (Chokdeemboon *et al.*, 2013). Several genetic variants of FGFR2 can cause Pfeiffer syndrome, however, only one mutation in FGFR1, the missense p.P252R alteration, has been reported in association with Pfeiffer syndrome (Muenke *et al.*, 1994). However, the presence of the p.P252R FGFR1 mutation is also found in healthy individuals (Chen *et al.*, 2016). The genetic study of Chen and colleagues (2016) found at least 8 rare, deleterious mutations in healthy individuals, indicating the capacity of certain genetic backgrounds to buffer the effects of disease-causing mutations.

Expressivity modifiers determine the severity of mutations. For example, a recent study in mice revealed the variable impact of craniofacial defects caused by mutations in *Sprouty* genes (Percival *et al.*, 2017). In particular, the FVB/NJ background was found to be more robust to *Sprouty* mutations than either 129X1/SvJ or C57BL/6J. This study also revealed that modifier genes can also change the direction of the effect, as the same loss of function mutation in *Spry1* caused opposite effects on craniofacial shape in two different inbred backgrounds (Percival *et al.*, 2017). Finally, pleiotropy modifiers determine the number of features that are affected by a mutation. Thus, genetic modifiers can also impact the spatial and temporal context of mutations (Mackay, 2014).

In most cases, the underlying mechanisms modifiying penotypic output of target gene mutations are unknown. However, some interesting examples have been elucidated. For example, craniosynostosis, the premature ossification of cranial sutures, can result from epistatic interactions between multiple members of the osteogenic differentiation pathway downstream of BMP signaling (Timberlake *et al.*, 2018). The BMP signaling cascade involves multiple activators and inhibitors upstream of osteogenic differentiation (Fig 2A). In a recent study investigating genetic causes of non-syndromic craniosyostosis, mutations in *SMAD6*, an inhibitor of BMP signaling, were identified (Timberlake *et al.*, 2016). The causative *SMAD6* mutations were often found in an unaffected parent, reflective of their incomplete penetrance. An earlier genome-wide association study had identified a craniosynostosis “risk allele” of *BMP2*. This risk allele harbors several single nucleotide polymorphisms associated with craniosynostosis within a non-coding region downstream *BMP2* (Justice *et al.*, 2012). Predicted transcription-factor binding sites in this region suggest it may be a cranial specific regulatory region increasing *BMP2* expression. Notably,

this *BMP2* variant is common and only very rarely causes craniosynostosis on its own (Komatsu and Mishina, 2016). However, when the *BMP2* risk allele occurs in association with *SMAD6* mutations, craniosynostosis occurs 100% of the time (Timberlake *et al.*, 2016). Similarly, a child with a severe case of craniosynostosis was found to have an *SMAD6* mutation (inherited from an unaffected parent) and a de novo *TCF12* mutation (Timberlake *et al.*, 2018). TCF12 heterodimerizes with TWIST1 to transcriptionally repress osteogenic genes downstream of BMP signaling (Fig. 2A).

Thus, the developmental process of suture ossification can be modified by several different types of genetic modifications, many of which involve multiple "hits." Allelic variation at the BMP2 locus among normal populations causes background differences in BMP2 expression. In an allelic background where BMP2 is high, a mutation reducing expression of a BMP inhibitor causes craniosynostosis. In this example, genetic alterations contributing to these epistatic interactions reduce gene expression through changes to cis-regulatory DNA sequences or through loss of function mutations within the protein coding sequence.

Genetic background may also explain inherited differences in methylation of regulatory elements, which can affect gene expression and disease penetrance. Zebrafish mutants in *mef2ca* exhibit variation in ectopic bone formation in their hyoid skeleton (Nichols *et al.*, 2016). The ectopic bone results from a switch in cell fate from ligament to bone. *mef2c* is a MADS domain-containing transcription factor regulating skeletal development (Miller *et al.*, 2007). Null *mef2ca* mutants exhibit low penetrance of ligament-to-bone transition However, the $mef2ca^{b1086}$ mutant allele, which is predicted to form a truncated protein with deleterious activity, exhibits variable fate switching (Nichols *et al.*, 2016). Penetrance of fate switching in $mef2ca^{b1086}$ mutants is heritable and strains with high penetrance express high levels of mutant transcript while low penetrance strains have low levels of expression. Further, levels of $mef2ca^{b1086}$ are associated with differences in methylation of an upstream transposable element that is thought to regulate its expression. Thus, the $mef2ca^{b1086}$ allele mediates cell fate changes in a Mendelian-like manner, yet it exhibits variable penetrance due to differences in epigenetic-mediated expression levels.

In isolation, methylation differences would be considered epigenetic modifications. However, Nichols and colleagues (2016) argue that an as yet unidentified genetic variant is ultimately responsible for the inherited difference in methylation. Differences in methylation at risk loci for cleft lip and/or palate have also been associated with variation in penetrance (Alvizi *et al.*, 2017). These data suggest that allelic differences in genes regulating methylation could be an under-appreciated mechanism contributing to variation in phenotypic penetrance.

Gene-environment interactions

Gene-environment interactions may be defined where one allele (polymorphism) responds differently to an external factor (Durham *et al.*, 2017). In humans, external factors including maternal nutritional status, diabetes/obesity-related conditions, and exposure to medications and/or environmental toxins are known to affect the incidence of craniofacial disease (Zhu *et al.*, 2009). The exact molecular mechanisms by which environmental factors influence phenotypes are not well established and may be difficult to study in human populations.

However, some insights on teratogenic mechanisms have been elucidated from studies in animal models, especially the effects of ethanol on holoprosencephaly (Fig. 2B).

Holoprosencephaly (HPE) is a highly variable congenital anomaly characterized by defects in midline patterning. Mutations in gene members of the SHH pathway are implicated in HPE, however, up to one-third of mutation carriers do not exhibit a clinical phenotype, and mutations found in many HPE patients are inherited from unaffected parents (Roessler and Muenke, 2010; Solomon *et al.*, 2012). Genetic modifiers, including the SHH co-receptors BOC and GAS1, contribute to the complex etiology of HPE (Hong *et al.*, 2017; Seppala *et al.*, 2014). However, environmental factors are also implicated. In particular, ethanol is an HPE-inducing teratogen, and studies of prenatal ethanol exposure in animal models have consistently shown that ethanol contributes to HPE by disrupting SHH signaling (Ahlgren *et al.*, 2002; Hong and Krauss, 2012, 2017; Li *et al.*, 2007).

In mice, homozygous mutations in *Cdo*, another Shh co-receptor, produce HPE phenotypes with background-specific phenotypic severity (Hong and Krauss, 2012). In the 129S6 background, $Cdo^{-/-}$ mice display low penetrance of HPE that can be exacerbated by either genetic or environmental factors. For example, the additional loss of one allele of *Shh* or *Boc* causes severe HPE phenotypes (Tenzen *et al.*, 2006). Similarly, ethanol exposure exacerbates defects in midline patterning and SHH signaling in 129S6 $Cdo^{-/-}$ mice (Hong and Krauss, 2012; Kahn *et al.*, 2017). The precise mechanism by which ethanol perturbs SHH signaling remains unknown. However, it has been hypothesized that ethanol directly inhibits CDO activity (Kahn *et al.*, 2017). It has also been hypothesized that ethanol reduces SHH signaling by blocking cholesterol modification of SHH (Li *et al.*, 2007). These data suggest that individuals with mutations in genes involved in SHH signaling may be particularly susceptible to embryonic ethanol exposure (Hong and Krauss, 2012).

Developmental stochasticity

Embryogenesis is the process by which a single cell generates all the cellular and tissue diversity within an organism from the same, shared genome. Despite this tremendous power to generate variation in gene expression and cell fate, development typically produces robust phenotypes (Waddington, 1942). A variety of mechanisms have evolved to limit phenotypic variation and/or direct it within a developmental structure, such as a body plan. Nevertheless, random variation in developmental processes can cause subtle phenotypic variation, which can be observed in isogenic populations such as genetically identical, inbred littermate mice (Hallgrimsson *et al.*, 2009; Parsons *et al.*, 2008).

Studies in bacteria and yeast have shown that gene expression is noisy and that clonal populations of cells exhibit substantial molecular variation (Eldar *et al.*, 2009; Eldar and Elowitz, 2010). Such noise appears to be essential to many cellular activities, including diversification of cell fates as well as adaptive evolution (Oates, 2011). Molecular variation in isogenic cells may also be influenced by cell cycle state or differences in location related to other cells, signaling molecules, or extra-cellular matrix. Under normal developmental conditions, such molecular variation is typically buffered by tissue-level processes, producing only subtle craniofacial variation (Thornhill and Moller, 1997). However,

molecular noise may be especially relevant to phenotypic variation in the context of genetic mutations.

Cell fate decisions are mediated, in part, by transcription factor expression and loss of function mutations in key transcription factors can cause alterations to cell fate decisions. For example, in the developing zebrafish head, *barx1* regulates the decision to become a joint cell versus a cartilage cell v(Nichols *et al.*, 2013), and *mef2ca* controls ligament versus bone cell fate decisions (Nichols *et al.*, 2016). A threshold model has been proposed to explain these alternative cell fate decisions, where the binary choice between cell fates depends upon whether or not sufficient protein levels are achieved to activate one cell fate over a default cell fate (Nichols *et al.*, 2016; Oates, 2011). Therefore, mutations that reduce protein levels such that they are at or near the threshold will produce heterogeneity in cell fate decisions as stochastic variation results in some cells reaching the threshold and others not. Variation in disease severity is the consequence of tissue-level responses to heterogeneity in single cell behavior (Oates, 2011). Thus, developmental stochasticity may explain both discrete cellular variation and continuous morphological variation (Fig. 3C).

For example, mice with mutations in *Satb2* exhibit continuous variation in jaw size (Fish *et al.*, 2011). While complete loss of Satb2 function causes extreme micrognathia and cleft palate, $Satb2^{+/-}$ mice exhibit a variable reduction in jaw size. Notably, dentary length of $Satb2^{+/-}$ mice encompasses the range of variation between wild-type and homozygous mutants. Reduction in jaw size upon loss of Satb2 is associated with apoptosis of NCC progenitors (Britanova *et al.*, 2006). That is, Satb2 activation is required for NCC survival and differentiation. This implies that the average cellular protein level in heterozygous mice is near the threshold for Satb2 activation (Fig. 2C, upper panel). Thus, in $Satb2^{+/-}$ mice, minor random variation in Satb2 protein levels leads to cellular heterogeneity in Satb2 network activation, and cells failing to activate the Satb2 network undergo apoptosis (Fig. 2C, lower panel). Variation in jaw size in heterozygous mice is therefore associated with inter-individual variation in the number of cells that undergo apoptosis.

The threshold model results in a non-linear relationship between genotype and phenotype, which has previously been predicted to explain high levels of morphological variation in disease models (Hallgrimsson *et al.*, 2009; Marcucio *et al.*, 2011; Young *et al.*, 2010). Evidence for this non-linear model in craniofacial disease was recently presented for mutations affecting *Fgf8*, a critical regulator of facial development (Green *et al.*, 2017). In mice, reduction in *Fgf8* mRNA does not affect facial shape until *Fgf8* levels drop below 40% of wild-type expression. Importantly, molecular variance exhibited by mutant individuals is similar to that observed in wild-type individuals. That is, increased phenotypic variance in mutant individuals is not due to an increase in molecular noise, but rather due to the average levels of protein being at or near the threshold for activation (Fig. 2C, upper panel).

Evolution of developmental processes

Much of our discussion so far has focused on how alterations to developmental processes contribute to phenotypic variation in disease. It is worth considering if and how alterations to developmental processes underlying vertebrate diversification are similar or different from

those occurring in disease processes. As an example, we will consider evolutionary alterations to splicing patterns. Finally, we briefly discuss the evolution of mechanisms buffering phenotypic variation.

Splicing alterations in evolution and disease

Splicing patterns have rapidly diverged in vertebrate evolution, and likely had a more important role in species-specific phenotypes than do alterations to overall gene expression levels (Barbosa-Morais *et al.*, 2012). In a comparison of the jaws of six different species of cichlids, differences in splicing were found to be much higher than overall gene expression differences, suggesting that alterations to splicing may facilitate rapid divergence (Singh *et al.*, 2017). Increased alternative splicing has contributed to extensive proteomic diversity in mammals, and especially primates, relative to other clades (Barbosa-Morais *et al.*, 2012; Gueroussov *et al.*, 2017). For example, exon skipping is more common in human embryos compared to mouse, contributing to approximately double the number of isoforms generated per orthologous gene (Chen *et al.*, 2017).

Species-specific changes to splicing are mostly cis-directed, occuring as changes to splice recognition sites, however, evolution of trans-acting RNPs also played a critical role in generating proteome diversity (Barbosa-Morais *et al.*, 2012). In particular, “nucleic acid binding” genes were found to be among the most frequently associated genes with species-classifying splicing events. Notably, cis-mediated species-specific differences in splicing of RNPs preferentially affect disordered regions rather than nucleic acid binding domains (Barbosa-Morais *et al.*, 2012; Gueroussov *et al.*, 2017). Disordered domains lack stable 3D structures. Instead they undergo induced fit structural changes and, therefore, are flexible mediators of protein-protein interactions (Tompa *et al.*, 2015). Thus, mammalian-specific splicing events generate an increase in RNP diversity by retaining nuclei acid binding domians, but alternatively including disordered domains (Gueroussov *et al.*, 2017). In turn, RNP diversity contributes to increased complexity of splicing through variation in the formation of high-order protein assembiles on pre-mRNA (Fig. 3).

Mutations associated with spliceosomopathies are mostly thought to result in haploinsufficiency of the affected gene (see Table 2 and references therein). In most cases, the mutations occur in the protein coding region, but do so in a manner that is not thought to produce a functional protein, but rather reduce overall protein levels (e.g., Marques *et al.*, 2016). Similarly, tissue-specific splicing decisions often result from differences in levels or activity of splicing factors (Grosso *et al.*, 2008). Tissue-specific splicing can be mediated by tissue-specific expression of spliceosome-associated RNPs or by alterations to the levels of core U snRNPs (Grosso *et al.*, 2008; Pacheco *et al.*, 2006). Interestingly, reductions in levels of RNPs occurring as a consequence of a disease-causing mutation cause regulated shifts in splicing profiles. For example, knock-down of Rpl22l1 causes mis-splicing of *smad2*. In zebrafish, mis-splicing of *smad2* is mediated by exon 9 skipping; In mice, exons 7 and 8 are skipped. The resulting smaller mRNAs do not produce protein, leading to overall reduction in smad2 levels which subsequently contribute to defects in gastrulation (Zhang *et al.*, 2017). Notably, loss of Rpl22l1 function does not increase variation or randomize splicing

outcomes. Rather, a complete shift from exon inclusion to exon skipping occurs in the absence of Rpl22l1.

Several other recent investigations of disease-causing mutations in splicing factors have shown that dysregulation of splicing occurs as a shift in splicing patterns (e,g, from exon inclusion to exon skipping) in a limited set of genes, rather than global disruption to splicing. For example, mice with heterozygous mutations in *Chd7* exhibit multiple splicing alterations, including exon skipping, retained introns, and alternative splice sites, however, only 227 splicing events were modulated in these mutants (Belanger *et al.*, 2018). Therefore, the disease mechanism could be characterized as a shift from one regulated state to another regulated state. Thus, both the specific molecular mechanism (changes in splicing factor levels) and outcome (regulated shift in splicing patterns) occur in evolutionary and disease processes.

Evolution of buffering mechanisms

Cellular outcomes are often determined by multiple regulatory inputs (e.g., Fig. 2A). Such complexity in gene regulatory networks buffers transcriptional noise and can also often buffer alterations to transcription levels caused by a single mutation. In particular, the development of complex structures such as the craniofacial complex is robust to most heterozygous mutations (Loewe and Hill, 2010). However, mutations in some genes (most of those discussed above), generate variable disease phenotypes in the heterozygous state. This suggests that some genes are more susceptible to perturbation than others.

Several recently described disease models propose that proteins typically have a threshold level for activation with a range in which normal function occurs, which can be modeled as a non-linear curve (Fish, 2016; Green *et al.*, 2017; Nichols *et al.*, 2016; Young *et al.*, 2010). This non-linear model explains both increased phenotypic variance in disease resulting from genetic mutations that decrease protein levels, as well as why different genes may have different susceptibility to heterozygous mutations based on the position of the curves along the x-axis (Fig. 4A). The presence of proteins that have a lower threshold requirement for maintaining normal phenotypic outcomes may reflect the evolution of buffering mechanisms.

At least two possible mechanisms could contribute to buffering genetic mutations. Genetic alterations that increase the input on a gene of interest (increase positive regulation), may increase robustness to a loss of one allele (Fig. 4B). Similarly, alterations to other genes in the same gene regulatory network may buffer reductions in any one particular gene (Fig. 4C). The contribution of many genes to a single process may therefore be a mechanism for developmental robustness. Further, the complexity of genetic interactions regulating developmental processes explains the poor genotype-phenotype correlations of many diseases, where phenotypic defects are not the result of a single mutation, but rather result from the combination of several genes with additive effects (Manolio and Collins, 2009).

Conclusion

Recent research has described how developmental processes are key mediators of genotype-phenotype relationships. Multiple genes contribute to the regulation of relatively fewer developmental processes and phenotypic outcomes ultimately derive from the orchestration and interaction of these developmental processes. We have described how individuals carrying mutations in genes involved in mRNA splicing and ribosome biogenesis have similar craniofacial disease phenotypes. Based on recent investigations into the pathogenesis of these diseases, we argue that alternative splicing and ribosome biogenesis are related processes acting upstream of protein synthesis. Because these two processes utilize some of the same molecular resources, they have similar metabolic profiles. In particular, craniofacial defects associated with both spliceosomopathies and ribosomopathies result, at least in part, from apoptosis of pre-migratory NCC. These data are particularly relevant to clinical treatments and precision medicine. Understanding disease through affected developmental processes has the potential to focus treatments on cellular and metabolic outcomes of processes rather than attempting to treat each genetic mutation individually. Finally, disease and evolutionary phenotypes may result from similar alterations to developmental processes. Therefore, further investigation into how and why developmental processes vary will have significant impact on both disease and evolutionary mechanisms.

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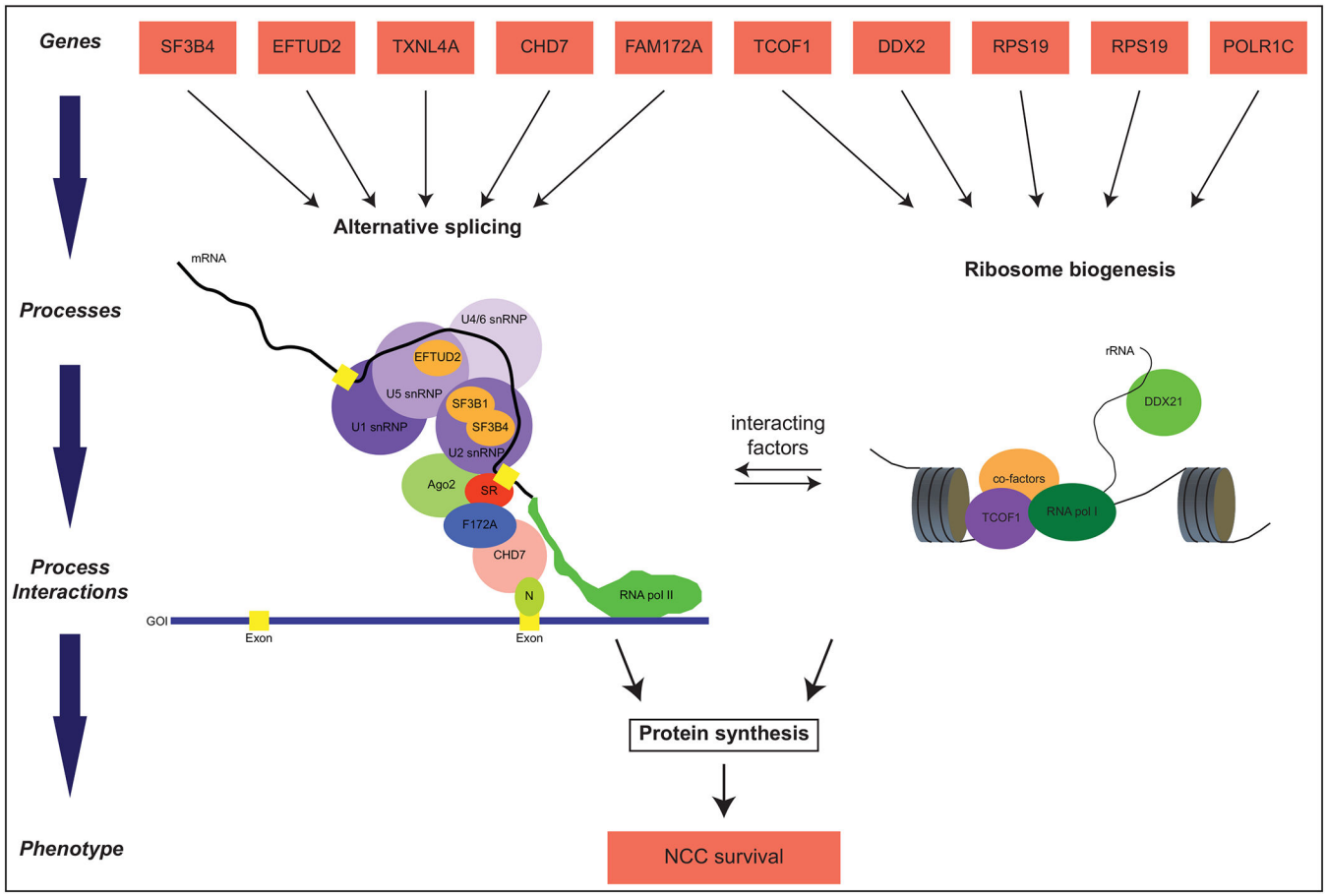

Figure 1: Developmental processes integrating ribosomopathies and spliceosomopathies

Relationships between genes and traits, shown in red boxes, are modeled to illustrate complexity and show processes integrating ribosomopathies and spliceosomopathies. Alternative splicing (left) and ribosome biogenesis (right) are two connected molecular processes upstream of protein synthesis. The spliceosomal small nuclear ribonucleoproteins (snRNPs), shown in purple, catalyze the splicing of exons, shown in yellow, in nascent mRNA molecules. Splicing factors associated with developmental defects are depicted as orange ovals inside their corresponding snRNPs. Other factors that link transcription and splicing are shown interacting with the spliceosome and RNA polymerase II. In the left panel, RNA polymerase I is shown synthesizing a strand of rRNA while interacting with TCOF1 and other co-factors. Both these molecular processes lead to protein synthesis, which is crucial for neural crest cell survival.

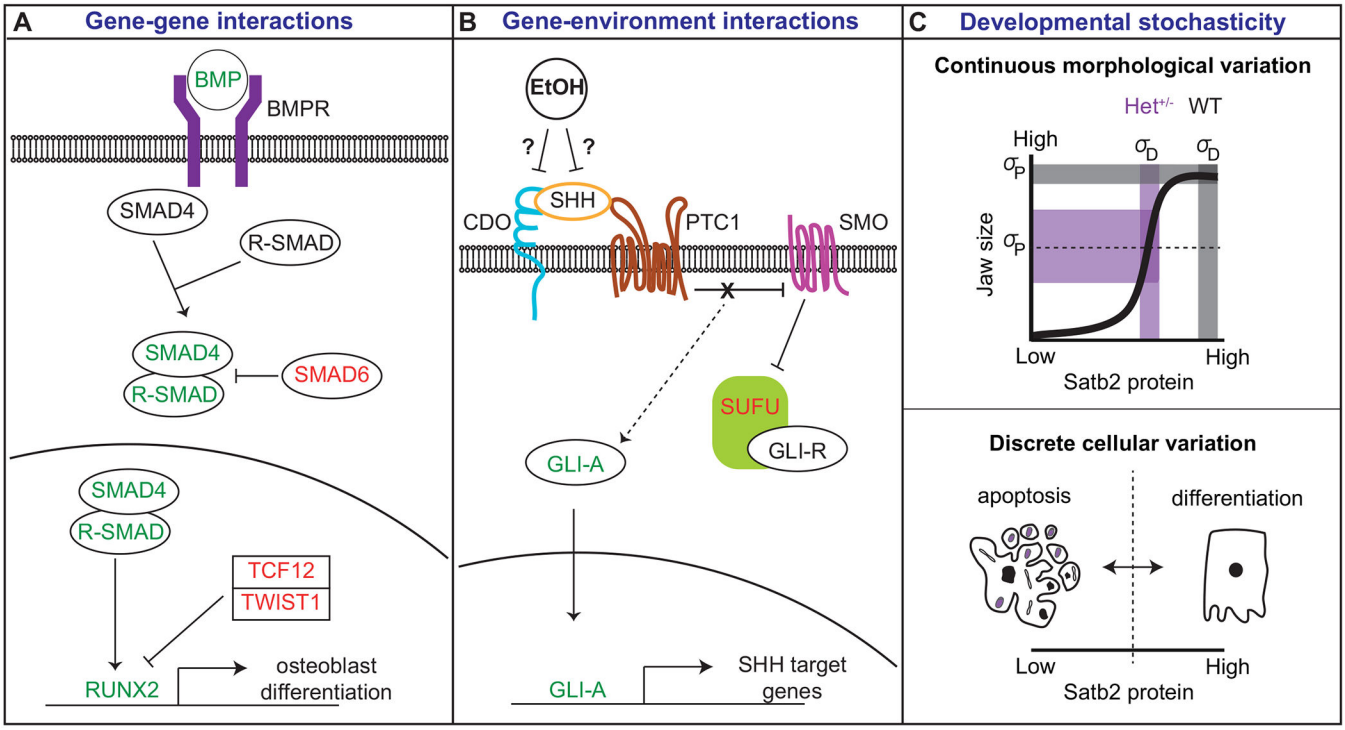

Figure 2: Mechanisms regulating variation in developmental processes

Factors contributing to variation in developmental processes include **A)** gene-gene interactions, **B)** gene-environment interactions, and **C)** developmental stochasticity. **A)** Gene-gene interactions are modeled by BMP signaling in osteogenesis. Proteins in red are negative regulators of the pathway; proteins in green are positive regulators. Heterozygous mutations in SMAD6, a negative regulator of the BMP pathway, may be buffered if they occur in isolation. However, if they occur in a "risk allele" background in which BMP levels are increased or a second negative regulator (e.g., TCF12) is decreased, disease phenotypes are observed. Image modeled after Timberlake *et al.* 2018. **B)** Gene-environment interactions are modeled by ethanol (EtOH) influences on SHH signaling. EtOH may exacerbate mutations in CDO, a SHH co-receptor, by negatively interacting with SHH binding to its receptors. Image modeled after Kahn *et al.* 2016. **C)** Developmental stochasticity is modeled by *Satb2*-mediated variation in jaw size. Satb2 protein levels have a non-linear relationship with jaw size, where wild-type and homozygous mutant individuals exhibit little population variance in size (grey shaded rectangles). However, heterozygous mutants are highly variable in size, encompassing the range of variation between wild-type and mutant (purple shaded rectangles). This continuous morphological variation (upper panel) can be explained by discrete cellular variation (lower panel). *Satb2*$^{+/-}$ cells are predicted to generate Satb2 protein levels that are at or near the threshold for Satb2 activation. Those cells that meet or surpass the threshold will proliferate and differentiate into osteoblasts; those cells that fall below the threshold will undergo apoptosis. Thus, random variation in the degree of heterogeneity in cell fate between individuals can explain variation in jaw size. Dotted lines indicate threshold for protein activity.

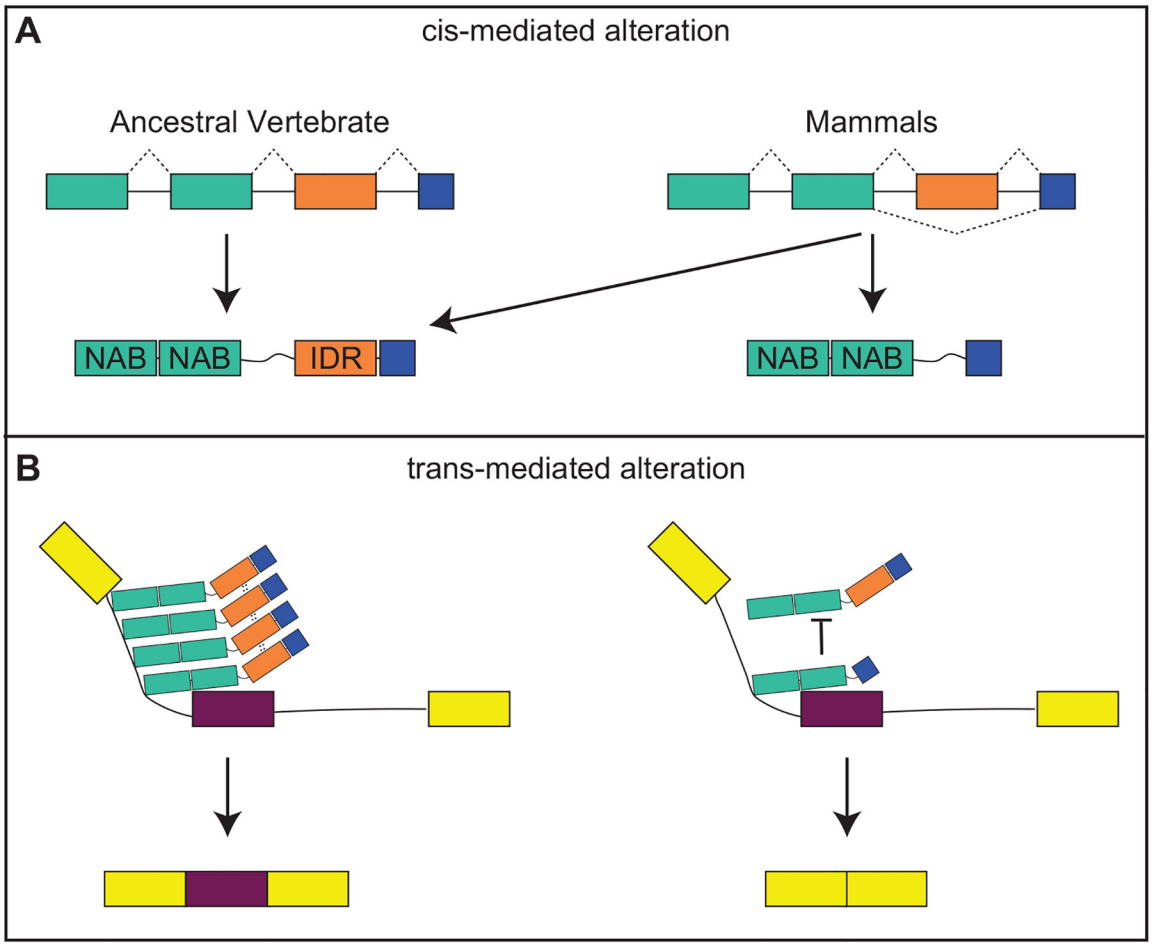

Figure 3: Mechanisms contributing to the evolution of splicing patterns

Both **A)** cis-mediated and **B)** trans-mediated alterations contribute to the evolution of splicing patterns. **A)** Mutations affecting splice recognition sites contribute to increased exon skipping in mammals. Such mutations are enriched around exons containing intrinsic disordered regions (IDR) and under-represented around nuclei acid binding domains (NAB). In this example, mammals are able to produce two protein isoforms from the same mRNA, one containing and IDR and one lacking the IDR. **B)** IDRs contribute to protein-protein interactions. A protein complex assembled among IDR-containing RNPs may promote exon inclusion (left). In the absence of IDRs, such complexes are not formed and exon skipping occurs (right). Image modeled after Gueroussov *et al.* 2017.

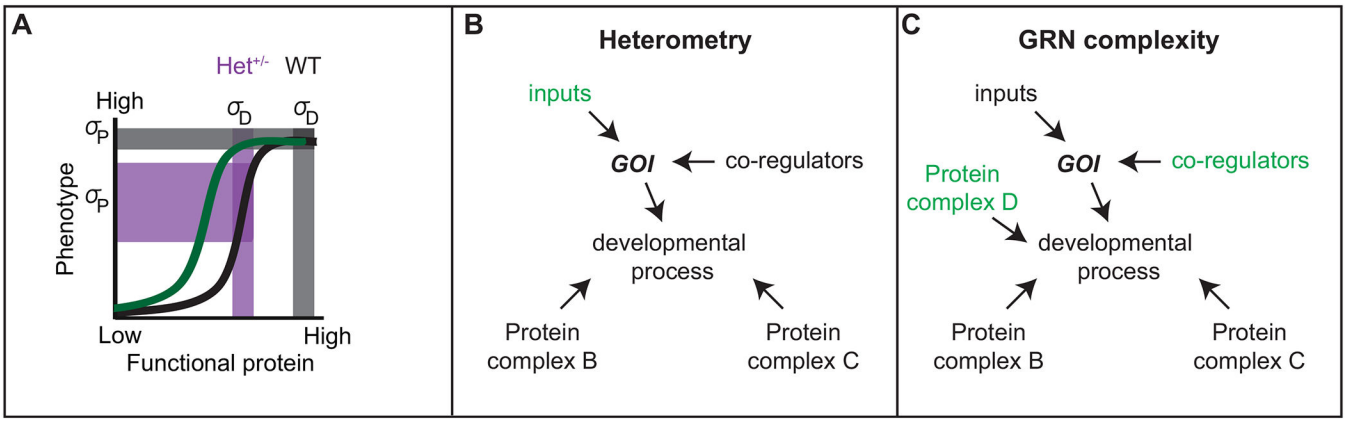

Figure 4: Evolution of buffering mechanisms

A) Non-linear model of genotype-phenotype relationships, where genotype is represented as functional protein produced by a gene (x-axis). Protein level variance is represented by the vertical bars; horizontal bars represent variation in phenotype. Note that, based on the threshold model, the same variance in protein levels has a significantly different effects on phenotype depending on protein levels relative to the threshold, where dark grey is wild-type (WT) and light gret is mutant ($Het^{+/-}$). Either heterometry or increases in GRN complexity may shift the position of a threshold value in a non-linear genotype-phenotype curve (black to green). **B)** Alterations to inputs regulating a gene of interest (GOI) may affect its levels (heterometry). **C)** Alterations to the number of co-regulators of protein complexes regulating a developmental process can increase gene regulatory network (GRN) complexity.

Table 1:

List of abbreviations used in the manuscript.

Abbreviation	Description
AFD	acrofacial dysostosis
EMT	epithelial-mesenchyal transition
HPE	Holoprosencephaly
IDR	intrinsic disordered region
LCLs	Lymphoblastic cell lines
MFD	mandibulofacial dysostosis
MFDGA	Mandibulofacial Dysostosis Guion-Almeida type
NCC	neural crest cell
POL	polymerase
RNP	ribonucleoprotein
rRNA	ribosomal RNA
TCS	Treacher Collins Syndrome

Table 2:

List of genes associated with spliceosomopathies and ribosomopathies, their disease phenotypes, and known molecular function.

Gene	Syndrome	Human Phenotype	References	Mutation	Function of Gene Product
SF3B4	1. Nager Syndrome 2. Rodriguez Syndrome	Craniofacial – malar and mandibular hypoplasia, down-slanting palpebral fissures, external ear defects and cleft palate. Limb – primarily hypoplastic or absent thumbs.	- Bernier *et al.*, 2012 - Petit *et al.*, 2013 - Champion-Arnaud and Reed, 2004	Frameshift or nonsense resulting in **haploinsufficiency**.	Member of SF3B complex of U2 snRNP involved in tethering U2 complex to branch site in pre-mRNA.
EFTUD2	Mandibulofacial Dysostosis, Guion-Almeida Type (MFDGA)	Craniofacial – overlaps with Nager syndrome and includes microcephaly. Limb – occasionally involves defects such as proximally placed thumbs and polydactyly of thumbs.	- Lines *et al.*, 2012 - Fabrizio *et al.*, 1997	Frameshift, nonsense, missense, deletions resulting in **haploinsufficiency**.	Encodes the spliceosomal GTPase U5–116kD, a member of the U5 snRNP.
TXNL4A	Burn-Mckeow Syndrome	Craniofacial – cleft lip and/or palate, short palpebral fissure, coloboma of the lower eyelids, prominent nasal bridge, and choanal atresia. Other – heart defects, large protruding ears.	- Wieczorek *et al.*, 2014 - Reuter *et al.*, 1999	Heterozygous deletion in the promoter region of TXNL4A.	Yeast ortholog of TXNL4A (Dib1) encodes essential component of the U4/U6-U5 tri-snRNP complex
EIF4A3	Richieri-Costa-Pereira Syndrome	Craniofacial – midline cleft mandible, cleft palate, glossoptosis, and micrognathia. Limb – limb reductions and clubbed feet.	- Favaro *et al.*, 2014 - Andreou and Klostermeier 2013	Expansion of 18–20 nucleotide motifs in the 5' UTR of EIF4A3.	Member of the exon junction complex (EJC). Anchors the EJC to the RNA.
SNRNPB	Cerebro-costo-mandibular Syndrome (CCMS)	Craniofacial – cleft palate, glossoptosis, and micrognathia. Rib defects – posterior gaps and missing ribs.	- Tooley *et al.*, 2016 - Lynch *et al.*, 2014 - Bacrot *et al.*, 2014 - Will and Lührmann, 2011	SNRPB codes for three splice variants, one containing an alternative exon that contains a premature stop and functions to auto-regulate protein levels. In CCMS, mutation in the splicing silencer region of the alternative exon increase its inclusion and result in lower levels of SmB and SmB'.	SNRNPB-encoded SmB and SmB' are splicing isoforms of one of the seven Sm proteins found in each snRNP.
CHD7 *FAM172A*	CHARGE Syndrome	**C**oloboma of the eye, **H**eart defects, **A**tresia of choanae, **R**etardation of growth and development, **G**enital abnormalities, and **E**ar anomalies. Craniofacial – temporal bone anomalies and cleft lip and/or cleft palate.	- Zentner *et al.*, 2010 - Vissers *et al.*, 2004 - Bajpai *et al.*, 2010 - Schulz *et al.*, 2014	Nonsense, missense, and single-copy 8q12 deletions of *CHD7*.	CHD7 is a chromatin remodeling factor that regulates the expression of key genes in the NCC GRN.

Gene	Syndrome	Human Phenotype	References	Mutation	Function of Gene Product
TCOF1, *POLR1C, POLR1D, DDX2*	Treacher Collins Syndrome (TCS)	Craniofacial – down-slanting palpebral fissures, micrognathia, facial bone hypoplasia, and cleft palate. Other – external ear defects, inner ear, and lower eyelid anomalies.	- Fazen *et al.*, 1967 - Phelps *et al.*, 1981 - Edwards *et al.*, 1997 - Dauwerse *et al.*, 2011 - Valdez *et al.*, 2004 - Gonzales *et al.*, 2005	Mutations in the *TCOF1* gene include splice site, missense, and nonsense mutations as well as insertions and deletions.	TCOF1, along with other factors, plays an important role in rDNA transcription and rRNA processing.
Ribosomal protein genes including: RPS19, RPS26, RPS27, RPL5, RPL11, GATA1	Diamond-Blackfan anemia (DBA)	Craniofacial – resemble defects observed in TCS. Limb – thumb abnormalities. Other – anemia caused by decrease of erythroid precursors.	- Delaporta *et al.*, 2014 - Kim *et al.*, 2012 - Gazda *et al.*, 2008 - Willing *et al.*, 1999 - Fylgare *et al.*, 2007 - Choesmel *et al.*, 2007 - Doherty *et al.*, 2010 - Sankaran *et al.*, 2012	Mutations in RPS19 include nonsense, missense, frameshift, and splice site mutations as well as deletions.	RPS19 is required for 18S rRNA synthesis and 40S ribosomal subunit maturation.