Structural Genome Variations Related to Craniosynostosis

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Keywords

Cranial sutures · Craniosynostosis · Functional networks · Skull shape malformations

Abstract

Craniosynostosis refers to a condition during early development in which one or more of the fibrous sutures of the skull prematurely fuse by turning into bone, which produces recognizable patterns of cranial shape malformations depending on which suture(s) are affected. In addition to cases with isolated cranial dysmorphologies, craniosynostosis appears in syndromes that include skeletal features of the eyes, nose, palate, hands, and feet as well as impairment of vision, hearing, and intellectual development. Approximately 85% of the cases are nonsyndromic sporadic and emerge after de novo structural genome rearrangements or single nucleotide variation, while the remainders consist of syndromic cases following mendelian inheritance. By karyotyping, genome wide linkage, and CNV analyses as well as by whole exome and whole genome sequencing, numerous candidate genes for craniosynostosis belonging to the FGF, Wnt, BMP, Ras/ERK, ephrin, hedgehog, STAT, and retinoic acid signaling pathways have been identified. Many of the cra-

niosynostosis-related candidate genes form a functional network based upon protein-protein or protein-DNA interactions. Depending on which node of this craniosynostosis-related network is affected by a gene mutation or a change in gene expression pattern, a distinct craniosynostosis syndrome or set of phenotypes ensues. Structural variations may alter the dosage of one or several genes or disrupt the genomic architecture of genes and their regulatory elements within topologically associated chromatin domains. These may exert dominant effects by either haploinsufficiency, dominant negative partial loss of function, gain of function, epistatic interaction, or alteration of levels and patterns of gene expression during development. Molecular mechanisms of dominant modes of action of these mutations may include loss of one or several binding sites for cognate protein partners or transcription factor binding sequences. Such losses affect interactions within functional networks governing development and consequently result in phenotypes such as craniosynostosis. Many of the novel variants identified by genome wide CNV analyses, whole exome and whole genome sequencing are incorporated in recently developed diagnostic algorithms for craniosynostosis.

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Craniosynostosis results from perturbed maintenance of suture patency during cranial development producing recognizable patterns of skull shape malformations depending on which sutures are affected. The incidence of craniosynostosis is around 1 in 2,500 live births. Craniosynostosis occurs either as an isolated nonsyndromic phenotype, or may be part of a syndrome including anomalies of the eyes, nose, palate, hands, and feet as well as impairment of vision, hearing, and intellectual development. Syndromic forms of craniosynostosis, occurring in association with other physical anomalies and intellectual disability, comprise approximately 15% of the cases and follow mendelian rules of inheritance [Wilkie et al., 2010; Zollino et al., 2017]. In approximately 85% of the patients, craniosynostosis occurs sporadically due to de novo genome rearrangements or gene mutations [Timberlake and Persing, 2018]. The sagittal suture is affected in 40–60% of the cases, the coronal suture in 20–30%, and the metopic suture in around 10% [Wilkie et al., 2010; Panigrahi, 2011; Katsianou et al., 2016]. In the autosomal dominant Apert, Crouzon, and Pfeiffer syndromes, multiple sutures are affected, while craniosynostosis in Saethre-Chotzen, Muenke, and craniofrontonasal syndromes is limited to the coronal suture [Ko, 2016]. Candidate loci and genes for craniosynostosis have been identified with classical karyotyping, molecular cytogenetic techniques, such as FISH and array-CGH, genome wide linkage analyses, homozygosity mapping, candidate gene, and whole exome and whole genome sequencing (WES and WGS). For most of the thus identified candidate genes, intra-genic sequence variants were found.

A considerable number of the affected genes in patients with craniosynostosis belong to the FGF, Wnt, BMP, Ras/ERK, ephrin, hedgehog, STAT, and retinoic acid signaling pathways [Katsianou et al., 2016; Timberlake et al., 2017; Timberlake and Persing, 2018]. Around one-third of all craniosynostosis cases harbor a mutation in the *FGFR1*, *FGFR2*, *FGFR3*, and *TWIST1* genes [Roscioli et al., 2013]. Most are monoallelic base substitutions, gene disruptions or CNVs, which exert dominant effects by either haploinsufficiency, dominant negative partial loss of function, gain of function, or epistatic interaction [Wilkie et al., 2010; Twigg and Wilkie, 2015; Veitia et al., 2018]. Recently, disruption of topologically associated chromatin domains (TADs), resulting from either chromosome rearrangements or CNVs and probably provoking ectopic gene expression, have been implicated in craniosynostosis [Klopocki et al., 2011; Will et al., 2017]. Mutations in the *FGFR1*, *FGFR2* and *FGFR3* genes in particular elicit pleiotropic effects in clinically distinct

craniosynostosis disorders such as Apert, Crouzon, Jackson-Weiss, and Pfeiffer syndromes [Meyers et al., 1996; Katsianou et al., 2016]. This review will discuss gene-annotation-based enrichment and functional interaction network analyses of the genes implicated in craniosynostosis and molecular mechanisms for dominantly acting mutations. Particular emphasis will be laid on the various types of structural genome variation in relation to craniosynostosis.

Craniosynostosis Gene Identification

The first clue towards a genetic cause for craniosynostosis emerged after karyotyping of sporadic patients with skull deformations, e.g., trigonocephaly, which revealed a small deletion of the terminal part of chromosome 7 [Gong et al., 1976; Dhadial and Smith, 1979]. Subsequent karyotyping with improved resolution of other patients allowed to exclude band 7p15 and to subsequently narrow down the locus for craniosynostosis to subband 7p21.1 [Bianchi et al., 1981; Fryns et al., 1985; Marks et al., 1985; Motegi et al., 1985; García-Esquivel et al., 1986; Schömig-Spangler et al., 1986; Speleman et al., 1989; Zackai and Stolle, 1998]. Similarly, loci for craniosynostosis on chromosome arms 2q, 5q, 9p, 11q, 14q, 17p, 17q, and 19p have been identified [Rutten et al., 1978; Lippe et al., 1980; Fryns et al., 1986; Stratton et al., 1986; Lucas et al., 1987; Lewanda et al., 1995; Thomas et al., 1996; Lemmyre et al., 1998; Shiihara et al., 2004; Lyon et al., 2015]. These findings indicate that several genes may be involved in regulating proper closure of cranial sutures during development.

Apart from appearing in sporadic patients, craniosynostosis also occurs as part of a familial syndrome with mostly dominant inheritance. This permitted to identify loci for craniosynostosis by linkage analyses in pedigrees affected with Crouzon, Pfeiffer, Antley-Bixler, and Jackson-Weiss syndrome [Jabs et al., 1994; Li et al., 1994; Muenke et al., 1994; Meyers et al., 1995; Park et al., 1995; Rutland et al., 1995; Schell et al., 1995; Chun et al., 1998; Reardon et al., 2000]. With restriction fragment length polymorphisms and microsatellite markers, significant linkage for Saethre-Chotzen syndrome with loci in chromosome bands 7p21.3 up to 7p14.3 (genome positions ranging from 11.6 Mb to 29.9 Mb) was found (Fig. 1; online suppl. Table 1; for online suppl. material see www.karger.com/doi/10.1159/000490480) [Brueton et al., 1992; Lewanda et al., 1994a, b; Rose et al., 1994, 1997; van Herwerden et al., 1994]. The wide genomic range of these loci

Fig. 1. Linkage analyses for Saethre-Chotzen syndrome. On top the ideogram of chromosome 7 with the region ranging from 10 to 30 Mb and the markers with significant LOD scores and their neighboring genes depicted underneath. The colored bars indicate the

linkage intervals for the different studies: Black: Brueton et al., 1992. Orange: Lewanda et al., 1994a. Blue: van Herwerden et al., 1994. Pink: Rose et al., 1994. Green: Lewanda et al., 1994b. Red: Rose et al., 1997.

with significant linkage may indicate locus heterogeneity. On the other hand, translocation breakpoint mapping of 8 unrelated patients localized the gene for Saethre-Chotzen syndrome within chromosome bands 7p21.1 and 7p21.2 [Reardon et al., 1993; Rose et al., 1994; Wilkie et al., 1995].

The gene for Saethre-Chotzen syndrome has been identified by several clues. First, in the mouse, the basic helix-loop-helix transcription factor *Twist* is mainly expressed in undifferentiated cells committed to muscle and cartilage development and governs cranial neural tube and head morphogenesis [Chen and Behringer, 1995; Fuchtbauer, 1995]. Second, the human *TWIST1* gene maps to the same chromosome band as the gene for Saethre-Chotzen syndrome, 7p21 [el Ghouzzi et al., 1997; Howard et al., 1997]. In patients with Saethre-Chotzen syndrome, missense, insertion, and deletion mutations within the basic DNA binding, helix I and loop domains of *TWIST1* were discovered, and nonsense mutations re-

sulted in premature termination of the protein [el Ghouzzi et al., 1997, 1999; Howard et al., 1997; Rose et al., 1997; Johnson et al., 1998; Paznekas et al., 1998]. In *Drosophila*, *Twist* affects the transcription of fibroblast growth factor receptors (FGFRs), which have previously been implicated in human craniosynostosis [el Ghouzzi et al., 1997; Howard et al., 1997]. In a patient with Saethre-Chotzen syndrome, an apparently balanced t(6;7)(q16.2;p15.3) translocation, located approximately 5 kb 3' of the *TWIST1* locus, produced a deletion of 518 bp of chromosome 7 [Krebs et al., 1997]. This indicates that loss of function of one *TWIST1* allele by a positional effect may be sufficient to provoke Saethre-Chotzen syndrome [Krebs et al., 1997]. In *Twist* heterozygous mutant mice, duplications of a metatarsus and phalanges, akin to some of the skeletal dysmorphisms in Saethre-Chotzen patients, have been observed. This prompted el Ghouzzi et al. [1997] to propose overossification of interparietal bones as a novel pathophysiological mechanism for hu-

man craniosynostoses. In a patient with features reminiscent of Saethre-Chotzen syndrome, a *de novo* chromothripsis event, involving chromosomes 1, 3, 7, and 12, and disrupting the *FOXP1*, *DPYD*, and *TWIST1* genes, has been described [Kloosterman et al., 2012]. The case of *TWIST1* as a gene implicated in Saethre-Chotzen syndrome turned out to be paradigmatic for some of the mutational mechanisms underlying craniosynostosis and other perturbation of bone organization during development and spurred a range of functional studies [this issue].

Based on the draft sequence of the human genome, arrays of uniquely localizing DNA probes, be it bacterial artificial chromosomes, oligonucleotides or SNPs, have been developed and used to map CNVs in individual genomes [Visser et al., 2003]. Depending on the spacing of the probes, losses or gains encompassing a single or several full genes or even a single exon can be detected [Coe et al., 2007; Boone et al., 2010]. Applying this technique to patients with craniosynostosis, CNVs involving the *GLI3*, *IHH*, *MMP23*, *RUNX2*, *SKI*, and *TCF12* genes were identified [Gajec et al., 2005; de Pater et al., 2006; Meford et al., 2010; Hurst et al., 2011; Klopocki et al., 2011; Carmignac et al., 2012; Doyle et al., 2012; Lee et al., 2013; Goos et al., 2016; Dinçsoy et al., 2017]. Such CNVs alter the dosage of putative candidate genes and thus affect the phenotype of the carrier by a dominant mechanism. The dosage of the encoded protein is critical if this serves as a receptor for a growth factor or another soluble molecule, in a signaling pathway, or as a transcription factor binding to a specific DNA target [Veitia and Birchler, 2010; Poot et al., 2011; Poot and Haaf, 2015; Veitia et al., 2018]. If a CNV contains several genes, a contiguous gene syndrome arises that often encompasses a highly complex set of phenotypes being the outcome of several different altered forms of gene action (see below). On the other hand, a deletion of a gene may provoke a phenotype that is distinct from that of a point mutation in the same gene. This is illustrated by patients with Saethre-Chotzen syndrome with *TWIST* deletions who present with intellectual disability in addition to the classical phenotypes of the syndrome [Cai et al., 2003].

More recently, techniques have been developed which allow sequencing all exons of all genes or the entire genome at once, termed whole exome sequencing and whole genome sequencing (WES and WGS, respectively). Performing WES on a patient and unaffected relatives allows identifying all single nucleotide variants (SNVs) without requiring previous linkage analysis. Thus, WES and WGS represent methods for genome-wide mutation

analysis at the nucleotide level. Since the genome of any individual harbors on average 30–100 *de novo* SNVs in comparison with the genomes of his or her parents, mere cosegregation of a SNV with a disorder within the family is not sufficient for the SNV to be considered pathogenic. To gauge their potential pathogenicity, complex bioinformatic processes based on evolutionary features and population distribution of the SNV in question and its effect upon the encoded protein have been developed. In addition, databases of large sequencing efforts are consulted to search for SNVs that are believed to be phenotypically neutral since they were detected in healthy individuals. This strategy should be used with caution since some of the databases contain SNVs that occur as somatic mosaics [Carlston et al., 2017]. By WES and WGS of large cohorts of patients with specific forms of craniosynostosis, be it as an isolated phenotype or as part of a syndrome, a considerable number of novel candidate genes and mutations have been uncovered [Roscioli et al., 2013; Miller et al., 2017; Timberlake et al., 2017; Timberlake and Persing, 2018]. Thus, *de novo* mutations in inhibitors of the WNT, BMP, and Ras/ERK signaling pathways in patients with nonsyndromic midline craniosynostosis and in the *IL11RA*, *EFNB1*, *TWIST1*, *CDC45*, *MSX2*, *ZIC1*, *FBN1*, *HUWE1*, *KRAS*, *STAT3*, *AHDC1*, and *NTRK2* genes have been documented [Roscioli et al., 2013; Miller et al., 2017]. In cases with nonsyndromic midline craniosynostosis, a digenic mechanism, involving concurrent presence of risk alleles of *BMP2* and *SMAD6*, has also been discovered [Timberlake et al., 2016]. Also concomitant derepression of *GLI3* and activation of *IHH* targets has been demonstrated [Veistinen et al., 2017]. Thus, molecular diagnosis of craniosynostosis beyond Saethre-Chotzen, Crouzon, Pfeiffer, Apert, and Jackson-Weiss syndromes based on mutations in the *TWIST1*, *FGFR1* and *FGFR2* genes has been complemented with a widening spectrum of loci and genes (Table 1) [Jabs et al., 1993, 1994; Muenke et al., 1994; Reardon et al., 1994; Park et al., 1995; Rutland et al., 1995; Wilkie et al., 1995; Wieland et al., 2004; Roscioli et al., 2013; Twigg and Wilkie, 2015; Miller et al., 2017; Timberlake et al., 2017; Timberlake and Persing, 2018].

The genetics of disturbed closure of cranial sutures does not adhere to classical assumptions, such as one gene, one phenotype. To the contrary, mutations in one gene, i.e., *FGFR2*, may elicit one out of several syndromes, i.e., Antley-Bixler, Apert, Crouzon, Jackson-Weiss, or Pfeiffer syndrome (Table 1). On the other hand, disturbed closure of one suture, i.e., the coronal suture, implicates several loci and genes: *CDC45*, *EFNB1*, *FGFR3*, *MSX2*,

Table 1. Craniosynostosis loci and candidate genes by method of discovery, type of disorder, and affected sutures

Method of discovery	Locus or gene	Disorder (isolated or syndromic)	Inheritance	Affected suture	References
Cytogenetics	7p21.1 (<i>TWIST1</i>)	Saethre-Chotzen syndrome	AD	coronal	Reardon et al., 1993; Rose et al., 1994; Wilkie et al., 1995; Krebs et al., 1997
	5q33.3 (<i>MSX2</i>)	unclassified syndromic	inherited translocation	sagittal, lambdoid	Shiihara et al., 2004
Linkage	<i>EFNB1</i>	craniofrontonasal syndrome	XLD	coronal	Twigg et al., 2004; Wieland et al., 2004
	<i>FGFR1</i>	Pfeiffer syndrome	AD	multiple	Muenke et al., 1994
	<i>FGFR2</i>	Antley-Bixler syndrome	AR	coronal, lambdoid	Chun et al., 1998
		Apert syndrome	AD	lambdoid	Wilkie et al., 1995
		Crouzon syndrome	AD	coronal	Rutland et al., 1995; Wilkie et al., 1995
		Jackson-Weiss syndrome	AD	unspecified	Park et al., 1995
	<i>FGFR3</i>	Pfeiffer syndrome	AD	multiple	Rutland et al., 1995
		Crouzon syndrome	AD	coronal	Meyers et al., 1995
		Muenke syndrome	AD	coronal, sagittal, lambdoid	Rannan-Eliya et al., 2004
	<i>MSX2</i> <i>POR</i>	unclassified syndromic Antley-Bixler syndrome	AD AR	not indicated coronal, lambdoid	Jabs et al., 1993 Adachi et al., 2004; Flück et al., 2004
Genome-wide CNV analysis	<i>RAB23</i> <i>TWIST1</i>	Carpenter syndrome Saethre-Chotzen syndrome	AR AD	multiple coronal	Jenkins et al., 2007 el Ghouzzi et al., 1997; Howard et al., 1997
	<i>GLI3</i>	Greig cephalopolysyndactyly syndrome	AD	metopic	Hurst et al., 2011
	<i>IHH</i> <i>MMP23B</i>	craniosynostosis with syndactyly frontal bossing/microcephaly	dn dn	sagittal metopic, sagittal, coronal	Klopocki et al., 2011 Gajecka et al., 2005; de Pater et al., 2006
	<i>RUNX2</i>	metopic craniosynostosis	dn	metopic	Mefford et al., 2010; Lee et al., 2013
	<i>SKI</i>	Shprintzen-Goldberg syndrome	AD	coronal, sagittal	Carmignac et al., 2012; Doyle et al., 2012
	<i>TCF12</i>	unclassified syndromic	AD	multiple	Goos et al., 2016; Dinçsoy et al., 2017
	<i>TWIST1</i>	Saethre-Chotzen syndrome	AD	coronal	Cai et al., 2003
Homozygosity mapping	<i>COLEC11</i>	3MC syndrome	AR	metopic	Rooryck et al., 2011
	<i>MASP1</i>	3MC syndrome	AR	metopic	Rooryck et al., 2011
	<i>IL11RA</i>	unclassified syndromic	AR	pansynostosis	Nieminen et al., 2011
Candidate gene sequencing	<i>KRAS</i>	Noonan syndrome type 3	AD	sagittal	Schubbert et al., 2006
WES and WGS	<i>AHDC1</i>	unclassified syndromic	dn	coronal, metopic	Miller et al., 2017
	<i>ARAP3</i>	nonsyndromic	dn	metopic	Timberlake et al., 2017
	<i>ASXL1</i>	Bohring-Opitz syndrome	dn, AD	metopic	Hoischen et al., 2011
	<i>ASXL3</i>	unclassified syndromic	dn	metopic	Dinwiddie et al., 2013
	<i>AXIN1</i>	nonsyndromic	dn	sagittal	Timberlake et al., 2017
	<i>B3GAT3</i>	nonsyndromic	dn	unspecified	Yauy et al., 2018
	<i>BMP2</i> and <i>SMAD6</i>	nonsyndromic	dn, AR	metopic, sagittal	Timberlake et al., 2016
	<i>CDC45</i>	Meier-Gorlin Syndrome	AR	coronal	Fenwick et al., 2016
	<i>DVL3</i>	nonsyndromic	dn	sagittal	Timberlake et al., 2017
	<i>EFNB1</i>	unclassified syndromic	dn	coronal	Miller et al., 2017
	<i>ERF</i>	unclassified syndromic	AD	multiple	Twigg et al., 2013
	<i>FBN1</i>	unclassified syndromic	dn	metopic, sagittal	Miller et al., 2017
	<i>HUWE1</i>	unclassified syndromic	dn	metopic	Miller et al., 2017
	<i>KAT6B</i>	Lin-Gettig or Say Barber Biesecker	AD	sagittal	Bashir et al., 2017
		Young Simpson syndrome			
	<i>KRAS</i>	unclassified syndromic	dn	pansynostosis	Miller et al., 2017
	<i>MEGF8</i>	Carpenter syndrome			Twigg et al., 2012
	<i>MESP1</i>	nonsyndromic	dn	sagittal	Timberlake et al., 2017
	<i>MSX2</i>	unclassified syndromic	dn	coronal	Miller et al., 2017
	<i>NPHP4</i>	nonsyndromic	dn	sagittal	Timberlake et al., 2017
	<i>NTRK2</i>	unclassified syndromic	dn	coronal	Miller et al., 2017
	<i>P4HB</i>	Cole-Carpenter syndrome	AD	coronal	Rauch et al., 2015

Table 1 (continued)

Method of discovery	Locus or gene	Disorder (isolated or syndromic)	Inheritance	Affected suture	References
	<i>PSMC2</i>	nonsyndromic	dn	metopic	Timberlake et al., 2017
	<i>PSMC5</i>	nonsyndromic	dn	metopic	Timberlake et al., 2017
	<i>RASAL2</i>	nonsyndromic	dn	sagittal	Timberlake et al., 2017
	<i>SMAD6</i>	nonsyndromic	dn	metopic, sagittal	Timberlake et al., 2017
	<i>SMO</i>	Curry-Jones syndrome	somatic mosaicism	coronal	Twigg et al., 2016
	<i>SMURF1</i>	nonsyndromic	dn	metopic	Timberlake et al., 2017
	<i>SPRY1</i>	nonsyndromic	dn	sagittal	Timberlake et al., 2017
	<i>SPRY4</i>	nonsyndromic	dn	sagittal	Timberlake et al., 2017
	<i>STAT3</i>	unclassified syndromic	dn	pansynostosis	Miller et al., 2017
	<i>TCF12</i>	unclassified syndromic	AD	coronal	Sharma et al., 2013
	<i>TWIST1</i>	unclassified syndromic	dn	metopic	Miller et al., 2017
	<i>WDR35</i>	Sensenbrenner syndrome	AR	sagittal	Gilissen et al., 2010; Bacino et al., 2012
	<i>ZIC1</i>	unclassified syndromic	AD	coronal	Twigg et al., 2015
	<i>ZIC1</i>	unclassified syndromic	dn	bilambdoid, sagittal	Miller et al., 2017
	<i>ZNF462</i>	unclassified syndromic	dn	metopic	Weiss et al., 2017

AD, autosomal dominant; AR, autosomal recessive; dn, de novo; WES, whole exome sequencing; WGS, whole genome sequencing; XLD, X-linked dominant.

NTRK2, *P4HB*, *TWIST1* (Table 1). Some of these genes are implicated in “classical” craniosynostosis syndromes, e.g., Saethre-Chotzen syndrome, while others relate to nonsyndromic craniosynostosis [Timberlake et al., 2017; Timberlake and Persing, 2018]. This highly complex constellation calls for a reconsideration of the current approaches to infer genotype-phenotype relationships. Concurrence of a gene disruption, or a CNV with a phenotype, in particular in sporadic cases, is no rigorous evidence for causal involvement. Even a, by convention statistically significant, cosegregation of an SNV with a phenotype in large kindreds or in cohorts of patients versus the general population is not sufficient to “prove” causality. In the following, a Gene Ontology (GO)-based enrichment strategy and conceivable regulation of development by gene interaction networks and possible molecular mechanisms of dominant gene action will be discussed.

Genes, Networks, and Biological Pathways

To evaluate possible functional relationships between these genes, the list in Table 1 has been analyzed with the STRING database [Snel et al., 2000; Szklarczyk et al., 2017]. This database appraises biologically meaningful physical and functional interactions by collecting experimental data on protein-protein interactions and using data on known pathways and protein complexes from curated databases. The protein-protein interaction predictions are derived from systematic co-expression analysis,

detection of shared selective signals across genomes, and automated text mining of the scientific literature [Snel et al., 2000; Szklarczyk et al., 2017]. The 14 genes discovered by cytogenetics, linkage, and genome-wide CNV analyses of sporadic and familial craniosynostosis cases form a functional network (Fig. 2) and fit into a limited number of biological processes. By GO enrichment analysis, 11 genes fit into the category of development (GO:0007275 with a Bayes factor of 13.09 and a Bonferroni corrected *p* value of 0.000028): *EFNB1*, *FGFR1*, *FGFR3*, *GLI3*, *IHH*, *MMP23B*, *MSX2*, *RUNX2*, *SKI*, *TCF12*, and *TWIST1*. Of these, *FGFR1*, *FGFR3*, *MSX2*, *RUNX2*, and *TWIST1* are specifically involved in skeletal development (GO:0001501 with a Bayes factor of 12.96 and a Bonferroni corrected *p* value of 0.000028).

Skeletal morphogenesis is modulated via the Ras and Rap1 signaling pathways as well as the actin cytoskeleton. Calvarial suture closure is primarily achieved by the *FGFR1*, *FGFR2*, and *FGFR3* genes, all encoding cell membrane receptors, which regulate distinct aspects of this process. Furthermore, the *TWIST1*-encoded basic helix-loop-helix transcription factor forms dimers with *TCF12*, which induce *FGFR2* expression and repress myogenesis by inhibiting the MyoD1 network [Sharma et al., 2013]. *TCF12*, among other activities, participates in the initiation of neuronal differentiation by binding to the *FGFR1*-encoded protein. *EFNB1*, which has been shown to interact with FGFRs and is thought to be involved in boundary formation at the coronal suture, constrains the orientation of longitudinally projecting axons and eye formation

Fig. 2. Functional network of craniosynostosis-related genes as identified by karyotyping, genome-wide linkage, and CNV analyses. Colored nodes represent genes and edges represent protein-protein associations. Pink: experimentally determined interactions. Light blue: interactions from curated databases. Black: interactions based on co-expression experiments. Light green: interactions inferred from text mining. Gray: protein homologies.

during early development by balancing FGF activities [Moore et al., 2004; Lee et al., 2009]. On the other hand, the transcriptional activity of STAT3 is enhanced after interaction with EFNB1 [Bong et al., 2007]. RUNX2 is an essential transcription factor for chondrocyte maturation and osteoblast differentiation, and in mice, mutated forms of FGFRs cause craniosynostosis and limb defects by upregulating *Runx2* expression [Komori, 2011]. Runx2, in turn, regulates *Ihh*, *PTHrP*, *Sox9*, *Col2a*, or *Col10a* gene expression in mice [Eswarakumar et al., 2004]. Thus, *IHH* is implicated in endochondral ossification by regulating the balance between growth and ossification of developing bones [Eswarakumar et al., 2004].

GLI3 is a zinc finger transcription factor with a dual function as transcriptional activator and repressor of the sonic hedgehog pathway and plays a role in regulating suture development by controlling the canonical Bmp-Smad signaling pathway [Tanimoto et al., 2012]. Apparently these genes form an intricate network, revolving around the FGFR system, in which they balance each other's activities. The cytochrome P450 oxidoreductase (*POR*), which controls the retinoic acid signaling pathway, and the matrix metalloproteinase 23B (*MMP23B*) genes are both outsiders. Both genes are related to non-autosomal dominant forms of craniosynostosis. The *POR* gene was mutated in the autosomal recessive Antley Bixler syn-

Fig. 3. Functional network of genes implicated in craniosynostosis as identified by karyotyping, genome-wide linkage, and CNV analyses, homozygosity mapping, candidate gene sequencing, WES, and WGS. For explanation of symbols, see Figure 2.

drome, while *MMP23B* has been deduced as the common denominator among de novo CNVs found in sporadic patients with defective suture closure [Gajecka et al., 2005; de Pater et al., 2006].

Adding the genes identified by homozygosity mapping and candidate gene sequencing and those by WES or WGS in patients with craniosynostosis further reinforces the notion of a network of interacting genes (Fig. 3). Of the 48 thus far identified genes, 28 form an interconnected network. In addition to the network centered around the *FGFR* genes in Figure 2, three more subnetworks formed by members of the Wnt, BMP, and Ras/ERK signaling pathways, comprising the *ARAP3*, *AXIN1*, *DVL3*,

MESP1, *PSMC2*, *PSMC5*, *RASAL2*, *SMAD6*, *SMURF1*, and *SPRY1* genes appear [Timberlake et al., 2016; Timberlake and Persing, 2018]. By GO enrichment analysis, 23 genes fit into the category of development (GO:0007275 with a Bayes factor of 16.82 and a Bonferroni corrected *p* value of 0.000672): *AXIN1*, *BMP2*, *DVL3*, *EFNB1*, *EGFL4*, *FBN1*, *FGFR1*, *FGFR3*, *GLI3*, *IHH*, *MMP23B*, *MSX2*, *NTRK2*, *RUNX2*, *SKI*, *SMO*, *SMURF1*, *SPRY1*, *SPRY4*, *STAT3*, *TCF12*, *TWIST1*, and *ZIC1*. Of these, *BMP2*, *FBN1*, *FGFR1*, *FGFR3*, *MSX2*, *RUNX2*, and *TWIST1* are specifically involved in skeletal development (GO:0001501 with a Bayes factor of 11.97 and a Bonferroni corrected *p* value of 0.000672). Thus, the genes discovered by classical

cytogenetics, linkage, and genome-wide CNV analyses (Fig. 2) as well as the larger set that has been obtained by including homozygosity mapping, candidate gene sequencing, WES, and WGS (Fig. 3) adhere to the same GO categories. This adds confidence that the findings of homozygosity mapping and the sequencing methods mentioned above are indeed relevant for the process of craniosynostosis.

Taking into account the temporal order of gene expression during cranial development, a pathogenetic framework has been proposed [Twigg and Wilkie, 2015]. Prenatally, stem cell specification and migration is controlled by genes of the sonic hedgehog pathway such as *RAB23*. Loss of both alleles of *RAB23* is either embryonically lethal or elicits the autosomal recessive Carpenter syndrome [Jenkins et al., 2007]. Subsequently, genes of the WNT signaling pathway, e.g., *ZIC1*, govern lineage commitment at the supraorbital center. A de novo loss of *ZIC1* has been observed in a patient with bilambdoid and sagittal craniosynostosis and gain-of-function mutations in *ZIC1* in 5 unrelated patients with coronal craniosynostosis and learning disability [Twigg et al., 2015; Miller et al., 2017]. Thereupon, the *EFBN1*, *TWIST1*, and *TCF12* genes drive suture boundary formation. Following, the *FGFR1*, *FGFR2*, and *FGFR3* genes of the RAS/MAK pathway, the *IHH*, *RAB23*, and *WDR35* genes of the Indian hedgehog pathway, in concert with the CYP26B1-encoding *POR* gene and *CDC45* regulate the balance between osteogenic proliferation and differentiation. Prenatally, the *ASXL1* and *COLEC11* genes coordinate neural crest specification, migration, and maturation [Twigg and Wilkie, 2015]. Thus, mutations or changes in expression level of any of the genes making up this extended craniosynostosis-related network engender a distinct craniosynostosis syndrome or set of cranial phenotypes.

Genotype-Phenotype Relationships

Establishing genotype-phenotype relationships for the genes implicated in craniosynostosis is far from straightforward. Patients with Apert, Crouzon, Jackson-Weiss, and Pfeiffer syndromes carry mutations dispersed throughout the genes of the *FGFR* family (Table 1), while mutations in the same domains of the *SMAD6* genes were found in patients with craniosynostosis affecting either sagittal, metopic, or both sagittal and metopic sutures [Meyers et al., 1996; Wilkie, 1997; Timberlake et al., 2016; 2017]. Mutations in the *FGFR1*, *FGFR2*, and *FGFR3* genes in particular prove to elicit pleiotropic effects (Table 1).

Similar mutations in exons IIIa and IIIc of *FGFR2*, which form the extracellular third immunoglobulin-like domain and adjacent linker regions, both involved in ligand binding, have been found in patients with Apert, Crouzon, Jackson-Weiss, and Pfeiffer syndromes [Meyers et al., 1996; Wilkie, 1997]. Based on the inter- and intrafamilial variability in the outcome of *FGFR2* mutations, the authors suggest that the clinically distinct Crouzon, Jackson-Weiss, and Pfeiffer syndromes are representative of a spectrum of related craniosynostotic and digital disorders [Meyers et al., 1996]. The S252W and P253R mutations in *FGFR2* account for the majority of Apert, Crouzon, and Pfeiffer syndrome cases, which all exhibit a characteristic Crouzon-like facial appearance, with protruding eyes, shortened face, and premature fusion of the coronal sutures [Katsianou et al., 2016]. Conceivably, these *FGFR2* mutations account for the core facial and cranial phenotypes of these syndromes, and other mutations and/or modifying or epigenetic factors may explain the distinguishing features of the affected patients.

A digenic model has been suggested as an explanation for some cases of the autosomal recessive Antley-Bixler syndrome and for patients with nonsyndromic midline craniosynostosis [Reardon et al., 2000; Timberlake et al., 2016, 2017]. For the latter, a common risk variant near the *BMP2* gene, which increased the penetrance of *SMAD6* mutations, was found with a genome wide association study [Timberlake et al., 2016, 2017]. The risk variant (SNP rs1884302, with a risk allele frequency of 0.34 and an odds ratio of 4.6) was overtransmitted to patients with de novo or transmitted mutations in *SMAD6* and other genes of the Wnt, BMP, and Ras/ERK signaling pathways [Timberlake et al., 2016, 2017]. This indicates a frequent 2-locus pathogenesis of nonsyndromic midline craniosynostosis and probably also rare syndromic craniosynostoses [Timberlake et al., 2017]. Thus, our understanding of congenital disorders with high locus heterogeneity can be advanced by using biological pathways inferred from gene mutations in patients with rare syndromic disorders [Timberlake et al., 2017].

This approach was extended to so-called RASopathies and 9p23p22.3 deletion cases as examples of conditions of intragenic mutations or CNVs provoking craniosynostosis [Zollino et al., 2017]. Trigonocephaly due to craniosynostosis in cases with 9p23p22.3 deletions probably has an oligogenic pathogenesis, involving the receptor-type protein tyrosine phosphatase (*PTPRD*) and the FRAS1-related extracellular matrix protein 1 (*FREM1*) genes [Visser et al., 2011; Mitsui et al., 2013]. Thus, 9p23p22.3 deletions represent an example of a contiguous gene syndrome.

Also in a heterogeneous group of mendelian disorders of chromatin modification in which the underlying genetic anomaly consists of disruption of one of the components of the epigenetic machinery, the so-called chromatinopathies, craniosynostosis was found. The targets of epigenetic modifications can be the DNA itself, e.g., cytosine methylation, or the proteins containing methyl-binding domains, involved in surveying or removing methyl groups in the DNA. Mutations in genes encoding these proteins bring about widespread downstream epigenetic consequences, ensuing phenotypic pleiotropy. The most frequent clinical manifestations thereof are intellectual disability, disorders of the neuronal migration, immune dysfunction, growth impairment, and skeletal anomalies. Craniosynostosis is a consistent, albeit rare feature in 4 out of 44 mendelian disorders of the epigenetic machinery [Bjornsson, 2015]. Conceivably, the phenotypic outcome in individual patients may reflect not only disruption of the compartments of the epigenetic machinery, but also the molecular constitution of the target genes [Paro, 1995; Law et al., 2010].

Craniosynostosis has been described in some, but not all patients with Kabuki syndrome, Koolen-De-Vries/KANSL1 haploinsufficiency syndrome, Bohring-Opitz syndrome, and KAT6B-related disorders. Craniosynostosis was found in about 6% of the patients with Kabuki syndrome [Armstrong et al., 2005; Topa et al., 2017]. In patients with 17q21.31 deletion (Koolen-De-Vries/KANSL1 syndrome), sagittal craniosynostosis, scaphocephaly, dolichocephaly, metopic ridges, bitemporal narrowing, trigonocephaly, brachycephaly, and frontal bossing have been described [Koolen et al., 2008, 2016; Sharkey et al., 2009; Dubourg et al., 2011]. Since also loss-of-function mutations in *KANSL1* (e.g., c.1652+2T>C; p.L552FfsX14) are sufficient to cause the full clinical phenotype associated with 17q21.31 deletions, it remains unclear whether craniosynostosis results only by haploinsufficiency for the genes in this deletion [Zollino et al., 2017]. In the Bohring-Opitz syndrome, trigonocephaly and craniosynostosis apparently reflect specific molecular properties of the causative gene, *ASXL1*, which is involved in activation and silencing of HOX genes and in chromatin remodeling [Hoischen et al., 2011]. In 90% of Bohring-Opitz syndrome patients with de novo heterozygous *ASXL1* mutations, mostly nonsense or truncating in nature, trigonocephaly was found [Hoischen et al., 2011; Magini et al., 2012; Dangiolo et al., 2015]. In summary, craniosynostosis may be a clinical phenotype of some chromatinopathies, albeit with a highly variable penetrance.

Disruption of Genes and Networks Entailing Dominant and Pleiotropic Gene Action

Except for the 3MC, Antley-Bixler, Carpenter, Meier-Gorlin, Sensenbrenner, and the unclassified IL11RA-related syndromes, craniosynostosis is part of a dominantly transmitted syndrome or results after a de novo structural genome variation or SNV. This means that monoallelic mutations or disruptions of a single chromosome elicit a phenotype by dominant action. Several molecular mechanisms for dominant gene action as relating to craniosynostosis have been proposed [Twigg and Wilkie, 2015]. Monoallelic loss of function of the DNA-binding or the dimerization regions of *TWIST1* or *ERF* elicit craniosynostosis via haploinsufficiency of the concerned gene. These mutations predominantly affect the glutamine 117 residue in *TWIST1* [Kim et al., 2017]. Although these mutations afford a modest biochemical effect on the encoded proteins, as reflected by Grantham matrix score of 98 and 121, they are sufficient to raise a phenotype in both humans and worms [Kim et al., 2017]. Haploinsufficiency as a pathogenetic mechanism depends on biallelic gene expression [Veitia, 2004, 2010; Birchler and Veitia, 2012]. For instance, upon a partial loss of function of one *STAT3* allele, due to a monoallelic substitution in the SH2 or DNA-binding domains, craniosynostosis results. Monoallelic substitutions precipitating increased ligand binding or constitutive receptor activation are examples of dominant effects of mutations found with the *FGFR1*, *FGFR2*, *FGFR3*, *IHH*, *MSX2*, and *ZIC1* genes [Twigg and Wilkie, 2015; Twigg et al., 2015]. Since these genes constitute nodes of a gene network (Fig. 3), these mutations disrupt the functioning of the network as a whole by a dominant mode of action.

Conceivably, SVs either affect the number of intact gene copies or disrupt the genomic architecture of a chromosome such that genes, parts of genes, and regulatory elements are relocated and their interactions disturbed [Poot et al., 2011; Poot and Haaf, 2015]. The first mechanism involves loss of a single allele by either gene disruption or formation of a CNV, which would be pathogenic if the requirements of the gene dosage balance hypothesis are met [Veitia, 2004, 2010; Birchler and Veitia, 2012]. The second mechanism disrupts TADs, which may alter gene expression levels instead of changing the structure of the encoded protein(s) [Reymond et al., 2007; Heinrichsen et al., 2009; Klopocki and Mundlos, 2011; Spielmann and Klopocki, 2013; Spielmann and Mundlos, 2013].

If one copy of a gene is lost due to disruption or CNV formation, and the gene is transcribed from both alleles, haploinsufficiency may ensue. Such a loss of one allele would be inconsequential if the gene product, i.e., the protein, functions as an isolated entity. Yet, in cases such as cell surface receptors, signal transduction pathways, transcription factors, ion channels, and regulation of chromatin conformation, proteins form complexes with other proteins or DNA [Veitia and Birchler, 2010]. If the relative amount of proteins making up these complexes is out of balance, due to not enough or too much of one of the participating proteins, the total amount of intact protein complexes will be insufficient for normal cell function. In this way, due to haploinsufficiency for a single gene, a dominant phenotypic effect arises, as has been observed in patients with epilepsy, intellectual delay, autism, and schizophrenia [Poot et al., 2011]. Following classical biochemical reasoning, the gene dosage balance hypothesis postulates that stoichiometric imbalances in macromolecular complexes and cellular networks may be responsible for abnormal phenotypes [Birchler and Veitia, 2012]. The outcome of protein disproportion due to gene dosage imbalance is probably more severe and more penetrant than that resulting from altered amino acid residue composition in a protein due to base substitution. Therefore, gene copy loss upon chromosome disruption or CNV formation should be considered as a separate entity.

In a cohort of 55 patients with Saethre-Chotzen syndrome, 6 (11%) had lost one copy of the *Twist1* gene [Cai et al., 2003]. These patients showed a 90% risk for developmental delay, which is 8 times higher than that for patients with base substitutions. Findings in 2 patients with large deletions close to *Twist1* suggested that haploinsufficiency of neighboring genes may also contribute to developmental delay in these cases [Johnson et al., 1998]. These observations are in agreement with the notion of a contiguous gene syndrome as a possible genomic cause for complex disorders, which include craniosynostosis as one of its phenotypes, such as Kabuki, 17q21.31 deletion (Koolen-De-Vries/KANSL1 syndrome), and 9p23p22.3 deletion syndrome [Koolen et al., 2008; Sharkey et al., 2009; Dubourg et al., 2011; Koolen et al., 2016; Topa et al., 2017; Zollino et al., 2017].

Improved resolution of CNV mapping by ever higher density arrays has resulted in detection of losses and gains of single exons or parts of exons [Boone et al., 2010]. Intragenic deletions of *CNTNAP2* , *IMMP2L* , *MCPH1* , and *NRXN1* have been found in patients with intellectual disability, speech delay, autism spectrum dis-

orders, hypotonia, Gilles de la Tourette syndrome, obsessive compulsive disorder, cortical dysplasia-focal epilepsy syndrome, Pitt-Hopkins syndrome, stuttering, attention deficit hyperactivity disorder, and schizophrenia [van Daalen et al., 2011; Schaaf et al., 2012; Poot, 2015, 2017]. Engineered forms of the *CNTNAP2* encoded Caspr2 containing internal deletions proved to be defective in oligomerization and thus exerted a dominant negative effect [Canali et al., 2018].

Intragenic losses and gains have been observed in several genes associated with craniosynostosis. During a screen of 39 patients with Crouzon, Jackson-Weiss, and Pfeiffer syndrome, one DNA insertion in exon IIIa of *FGFR2* in a Crouzon syndrome patient and one in exon IIIc in a Pfeiffer syndrome patient were found [Meyers et al., 1996]. In one patient with Apert syndrome, a 5' truncated Alu insertion into exon IIIc of *FGFR2* and in a second patient a 1.93-kb deletion, removing exon IIIc, were detected [Bochukova et al., 2009]. In another patient with Apert syndrome, a 1,372-bp deletion between *FGFR2* exons IIb and IIIc brought about a chimeric IIb/III exon [Fenwick et al., 2011]. In 2 patients with mild Crouzon syndrome-like facial features, 2 variants of *FGFR2* , c.1083A>G and c.1083A>T, precipitating loss of the normal donor splice site and the use of an alternative cryptic splice site were found [Fenwick et al., 2014]. This altered splicing led to an in-frame deletion of the terminal 17 amino acid residues of exon IIIc (p.Gly345_Pro-361del), including 4 residues that form specific contacts with the ligand FGF2 [Fenwick et al., 2014].

In a cohort of 55 patients with Saethre-Chotzen syndrome and other conditions with craniosynostosis, loss of the entire *Twist1* gene was found in 4 cases [Johnson et al., 1998]. Finally, in a cohort of 18 patients with coronal craniosynostosis and negative testing for *FGFR2* , *FGFR3* , and *Twist1* mutations, 3 patients harbored *TCF12* deletions of exons 7 through 18, 19, and 20, respectively [Goos et al., 2016]. A fourth patient carried a tandem duplication of exons 19 and 20. While these cases seem to be relatively rare, the considerable effort needed to uncover these indicates that intragenic SVs may be more common than hitherto thought. Given the phenotypic impact of deletions or duplications via probably a dominant negative mechanism, these variants merit due attention.

Until recently, position effects due to SVs have received little attention. The first to be reported in the context of craniosynostosis was a breakpoint of an apparently balanced t(6;7)(q16.2;p15.3) translocation approximately 5 kb 3' of the human *Twist1* gene in a patient

with a mild form of Saethre-Chotzen syndrome [Krebs et al., 1997]. A second report covered 2 patients with Saethre-Chotzen syndrome and a translocation or inversion with a breakpoint at 260 kb 3' from the *TWIST1* gene [Cai et al., 2003]. In patients with craniosynostosis and syndactyly, duplications of conserved noncoding elements (CNEs) associated with the *IHH* locus were observed [Klopocki et al., 2011]. These CNEs drove reporter gene expression according to a pattern similar to that of wild-type *Ihh* expression. The authors concluded that the observed duplications provoke misexpression and/or overexpression of the *IHH* gene and in this way affect complex regulatory signaling networks during digit and cranial development [Klopocki et al., 2011]. This study has been corroborated by the observation of a duplication at the *IHH* locus in a patient with acrocallosal syndrome [Bochukova et al., 2009]. Moreover, a 900-kb duplication involving the entire *IHH* locus was found to mimic acrocallosal syndrome [Yuksel-Apak et al., 2012]. In a cohort of 182 Spanish craniosynostosis patients, a duplication of the *IHH* regulatory region was discovered in a patient with craniosynostosis Philadelphia type, which underscores the importance of *IHH* disruptions for cranial and skeletal development [Paumard-Hernández et al., 2015]. In mice, *Ihh* proved to be a pro-osteogenic factor, positively regulating intramembranous ossification, while loss of *Ihh* reduced Bmp expression within the cranial bones [Lenton et al., 2011]. Transgenic reporter and genome-editing experiments in mice showed that *Ihh* expression is regulated by a cluster of CNEs consisting of at least 9 enhancers, each with individual tissue specificity in the digit anlagen, growth plates, cranial sutures, and fingertips [Will et al., 2017]. These enhancers function additively as consecutive deletions revealed growth defects of the skull and the long bones. In contrast, *Ihh* duplications, not only caused dose-dependent upregulation, but also misexpression of *Ihh*. The latter, led to abnormal phalanges, fusion of sutures, syndactyly. These findings demonstrate that precise spatiotemporal control of developmental gene expression in the skeletal and the cranial bones is achieved through complex multipartite enhancer ensembles. Alterations in the composition of such clusters by SVs may provoke gene misexpression and dysmorphic disorders. Collectively, these results show that SVs outside genes are able to disrupt TADs, which subsequently disturb tissue- and organ-specific patterns of gene expression eventually producing craniosynostosis and other types of dysmorphology.

Conclusion

Analyses of SVs in patients with craniosynostosis has aided in identifying candidate genes involved in cranial development and has provided valuable clues as to their function. The latter are being investigated in studies of model organisms (Lee et al., this issue). With a few exceptions, craniosynostosis ensues after de novo SVs and SNVs or by dominant inheritance. These mutations exert their effects by perturbing physical interactions of the altered protein with its cognate protein or DNA target. SVs disrupting TADs may alter gene expression levels or lead to ectopic gene expression. A significant fraction of the thus far identified candidate genes prove to be part of several interconnected functional networks. Depending on which node of these craniosynostosis-related networks is affected, a distinct craniosynostosis syndrome or set of phenotypes arises. Recent findings have raised the image of digenic inheritance in some cases of craniosynostosis, which calls for genome-wide investigation involving array-CGH, WES or WGS [Timberlake et al., 2017; Veistinen et al., 2017]. A molecular diagnosis of familial, syndromic craniosynostosis cases can be achieved through targeted sequencing of the genes identified by linkage analyses (e.g., *EFNB1*, *FGFR1*, *FGFR2*, *FGFR3*, *MSX2*, *POR*, *RAB23*, and *TWIST1*) [Kutkowska-Kaźmierczak et al., 2018]. Nonsyndromic cases of sagittal and/or metopic craniosynostosis should first be screened for *SMAD6* mutations, and nonsyndromic coronal craniosynostosis be investigated for *TCF12* and *TWIST1* mutations and the *FGFR3* P250R variant [Timberlake and Persing, 2018]. Cases of sporadic, nonsyndromic midline craniosynostosis negative for *TCF12* and *TWIST1* mutations and the *FGFR3* P250R variant as well as their parents should preferably be investigated by array-CGH, WES, or WGS [Zollino et al., 2017; Kutkowska-Kaźmierczak et al., 2018; Timberlake and Persing, 2018]. The thus found de novo CNVs and SNVs may then be prioritized for adhering to the GO category of skeletal development (GO:0001501) or the wider category of development (GO:0007275). On the other hand, the genes being part of the extended functional network depicted in Figure 3, merit further functional studies and may eventually represent targets for pharmacological intervention in patients with craniosynostosis.

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