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Running title: *MYCN* in Feingold syndrome and isolated oesophageal atresia

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ABSTRACT

Feingold syndrome (FS) is a syndromic microcephaly entity for which *MYCN* is the major disease-causing gene. We studied the expression pattern of *MYCN* at different stages of human embryonic development and collected a series of 17 FS and 12 isolated oesophageal atresia (IOA) cases. A *MYCN* gene deletion/mutation was identified in 47% of FS cases exclusively. We hypothesized that mutations or deletions of highly conserved non-coding elements (HCNEs) at the *MYCN* locus could lead to its misregulation and thereby to FS and/or IOA. We subsequently sequenced 5 HCNEs at the *MYCN* locus and designed a high-density tiling path comparative genomic hybridization array of 3.3 Mb at the *MYCN* locus. We found no mutations or deletions in this region, supporting the hypothesis of genetic heterogeneity in FS.

INTRODUCTION

Feingold syndrome (FS, MIM164280) combines characteristic digital anomalies (i.e. brachymesophalangy of the 2nd and 5th fingers and brachysyndactyly of the toes), microcephaly, oesophageal/duodenal atresia, and variable learning disabilities [1]. FS has been mapped to 2p23–24 [2] and is the consequence of *MYCN* gene (MIM 164840) loss-of-function either by germline deletions or coding sequence mutations [3,4]. Conversely, *MYCN* amplification is a prognostic factor for a bad outcome and found in about 10% of neuroblastoma [5].

In this study, we studied the expression pattern of *MYCN* at different stages of human embryonic development and screened a cohort of 17 patients suspected of FS and 12 cases with isolated oesophageal atresia (IOA). We identified a heterozygous mutation/deletion in 7

FS cases (47%) and no mutation or deletion in IOA. Some HCNEs, able to direct *N-myc* expression, have been identified in transgenic mice [6 7 8]. We hypothesized that deregulation of tissue- or stage-specific *MYCN* expression following mutation or disruption of regulatory highly conserved non-coding elements (HCNEs) at the *MYCN* locus could lead to FS and/or IOA. We subsequently sequenced 5 HCNEs at the *MYCN* locus and searched for small deletions in the 3.3 Mb vicinity of *MYCN*.

PATIENTS AND METHODS

A total of 29 patients were included in the study: 17 patients with possible FS (Table 1) and 12 patients with IOA. Diagnostic criteria for FS were the presence of three or more of the core features: i) microcephaly, ii) brachymesophalangy of the 2nd and 5th finger, iii) 2/3 or 4/5 toe syndactylies, and iv) oesophageal or duodenal atresia. Whereas post-natal microcephaly was constant after 3 years of age, head circumference was normal at birth in 3 cases. All patients showed mild to moderate mental retardation and eight developed postnatal growth retardation. Brachymesophalangy of the 2nd and 5th finger was noted in 15, syndactylies in 12 and oesophageal atresia in 14 of the 17 cases (Figure 1, Table 1). Additional features are listed in Table 1. All IOA cases were sporadic (10 type III and 2 type I) with no additional malformations.

Blood samples were obtained with informed consent and DNA was extracted according to standard protocols. DNA sequencing of the 3 coding exons and intronic flanking regions was performed by the fluorometric method on both strands [ABI BigDye Terminator Sequencing Kit V.2.1, Applied Biosystems]. Comparative genomics analysis of the *MYCN* locus indicated 5 highly conserved non-coding elements (HCNEs) with >75% identity over 350 bp across humans, rhesus, dog, and mouse (Figure 1). These HCNEs were studied by

direct sequencing in all patients with no coding sequence mutation (primers available on request).

A 3.3 Mb region extending 1.94 Mb centromeric (5') and 1.36 Mb telomeric (3') to *MYCN* (chr2:12,800,000-16,590,000; NCBI Build 36.1) was studied by fine-tiling array-based comparative genomic hybridization (CGH; NimbleGen Systems) on 6 FS patients and 10 IOA with no *MYCN* coding sequence (CDS) mutation as well as 550 normal-banded chromosomes on blood karyotype. The average spacing of probes in Nimblegen fine-tiling array is 52 bp. A deletion was considered when at least 10 probes were abnormal, giving a deletion detection resolution of about 500bp at the *MYCN* locus. Genome wide Array-CGH with a resolution of 50 kb was performed in the 5 FS patients with no *MYCN* mutation and normal Nimblegen fine-tiling array, using the Agilent Human Genome CGH Microarray Kit 244 K (Agilent Technologies, Santa Clara, CA).

To study MYCN expression during human development, embryos were collected from terminated pregnancies in agreement with French bioethics laws (94-654 and 04-800) and the Necker Hospital ethics committee. Probe synthesis and hybridization were carried out as described previously [9].

RESULTS

Direct sequencing and searching for deletion in *MYCN* locus

We identified a heterozygous coding sequence mutation in 7 patients (five novel, table1). All mutations resulted in a premature stop codon that removed the basic helix-loop-helix (b-HLH) and the leucine-zipper (LeuZ) domains or modified a conserved amino acid essential for DNA binding (figure 2). One patient had a deletion of 425Kb encompassing the

MYCN gene only. Six mutations occurred *de novo* and one was inherited from the affected father (AO28, Table 1) who showed brachymesophalangy of the 2nd and 5th fingers, syndactyly between the 4th and 5th toes, microcephaly and mild mental retardation. Additional features observed in patients harboring a *MYCN* coding sequence mutation or deletion were congenital heart malformations (2 cases), kidney hypoplasia (2 cases), asplenia (1 case) and diaphragmatic hernia (1 case). This last malformation had never been reported previously and CGH analysis showed no additional rearrangements in this patient. The *MYCN* locus was further investigated in patients with no coding sequence mutations; we sequenced 5 HCNE identified in the *MYCN* locus (Figure 2) and identified no nucleotide variations in either FS or IOA patients. Fine-tiling array-based CGH identified no micro-rearrangements in the 3.3 Mb region encompassing *MYCN*. Genome wide array-CGH 244 K was normal in the 5 patients with no *MYCN* CDS mutation and normal Nimblegen fine-tilling array (Table1).

MYCN expression in early human development

Additional features observed in patients with a MYCN mutation motivated the study of *MYCN* expression at different stages of human embryonic development (Figure 3). At Carnegie stage (CS) 13, *MYCN* appears ubiquitously expressed, with higher expression in the limb bud mesenchyme (Fig 2A-B). At CS15, *MYCN* is differentially and highly expressed in the CNS/PNS, the oesophageal and bronchic epithelia, Rathke's pouch, sympathetic ganglia, and both ectodermal and mesenchymal components of the forelimb. At CS17-18, *MYCN* is highly expressed throughout the CNS/PNS and in both Rathke's pouch and the corresponding precursor of the neurohypophysis, the infundibulum (Fig 3T-U), the smooth muscle of the umbilical arteries, the adrenal gland and the hindgut as well as other sites (Fig3Q-U).

However, despite low levels of cardiac expression seen *in situ* at C13, we no longer observed any cardiac expression at CS18 (Fig 3V-W).

DISCUSSION

We identified a *MYCN* mutation in 50% of our cases (8/17). No major phenotypic differences could be found among the core features of FS retrospectively, between patients with and without a *MYCN* mutation (Table 1). Only syndactyly of toes 4 and 5 was more frequent in the group with *MYCN* mutations. The high frequency of oesophageal atresia in our series is due to a recruitment bias through paediatric surgeons. Importantly, no additional malformations were present in the group of patients without mutations. Although head circumference can be normal at birth, post-natal microcephaly is constant in our series. Most patients were sporadic cases, contrasting with a previous report [4]. This discrepancy could be ascribed to both a recruitment bias for familial cases before the gene was identified, and the fact that clinically, the entity is better recognized since then. Several additional congenital malformations have been reported in FS; i.e, vertebral malformations, congenital cardiac defects and renal hypoplasia [4]. Renal hypoplasia needs to be detected early on in order to prevent renal failure [2]. One of our patients presented asplenia. This has not hitherto been reported in FS but is present in the *N-myc* hypomorphic mouse model [10]. A diaphragmatic hernia was detected in the same patient at birth. Facial features reported in FS are tenuous and combine short palpebral fissures, broad nasal bridge and micrognathia.

We studied the pattern of expression of *MYCN* at different stages of normal human embryonic development. *MYCN* is widely expressed in forelimb mesenchyme at the stages we studied, consistent with the constant distal bone malformations observed in FS. Expression in Rathke's pouch raises the question of involvement of the pituitary gland in the growth deficit. We observed *MYCN* expression in both bronchial tubes and the oesophagus at CS15, but not

in the diaphragm at CS17-18. *N-myc* knockout mice had been generated concomitantly by three independent groups [11] [12] [13]. Embryonic lethality was consistently observed between embryonic days E10.5 and E12.5 of gestation, with developmental delay and small size of mesonephros, lung, heart and gut. Interestingly, mutant mice with 25% of wild-type levels of N-myc protein die at birth and are unable to breathe, due to a severe deficiency in lung branching morphogenesis [10].

The molecular mechanisms underlying the regulation of *MYCN* expression have not been totally elucidated. It has been shown, by replacing endogenous *N-myc* coding sequences by the *c-myc* ones, that *c-myc* can complement *N-myc* functions [14]. Therefore, specificity of both genes resides in their controlled expression patterns. No mutation/deletion of *MYCN* regulatory elements could be identified in human. Altogether, these results are suggestive of genetic heterogeneity in FS.

Conflict of interest: The authors declare no conflict of interest.

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Legends to table and figures

Table 1: Clinical features in the series of 17 FS patients with and without *MYCN* mutation. M: male; F: female; SD; standard deviation, ASD: atrial septal defect, VSD: ventricular septal defect, AC: Aortic coarctation, MA: mitral atresia, del: deletion. NP: not performed. *The father and one sister of AO39 are microcephalic and have hands anomalies (brachymesophalangy of the 2^{nd} and 5^{th} fingers and brachysyndactyly of the toes). The sister has also learning disabilities. **The mother of AO41 is microcephalic and has hands anomalies (brachymesophalangy of the 2^{nd} and 5^{th} fingers).

Figure 1: Patient AO68 with Feingold syndrome and *MYCN* deletion. Note round face, brachymesophalangy of the 2^{nd} and 5^{th} finger, and short feet with brachydacty.

Figure 2: Schematic representation of the *MYCN* locus (6647 bp). The deletion and mutations identified in 5 FS patients and HCNEs with >75% identity over 350 bp across humans, rhesus, dog and mouse (ECR browser software) are shown.

Figure 3: *MYCN* expression in early human development.

Antisense and sense (negative control) riboprobes are presented side by side A to P in panels. **A**, At Carnegie stage (CS) 13 (37 days'gestation) *MYCN* is ubiquitously expressed. **C**, CS15 (36 days' gestation), transverse section in head, showing *MYCN* expression is still widespread but particularly strong in brain and craniofacial mesenchyme, as well as the precursors to the pituitary gland. **E**, CS15, transverse section through the cervical neural tube. **G**, CS15, section at level of forelimb. As compared with the control (**H**), no expression is observed in the liver but is specific to both epithelia and mesenchyme of bronchi and oesophagus (**I**-**J**) and forelimb bud (**M**-**N**) as well as at levels of the central and peripheral nervous system (CNS/PNS; **I**-**L**, **O**-**P**).

Q, Hematoxylin-eosin stain of sagittal section at CS17-18 (42-45 days'gestation). *MYCN* expression in an adjacent section (**R**) shows continued but diminished expression in the CNS/PNS and craniofacial mesenchyme, and presence of transcripts in the adrenal gland, hindgut and genital tubercule. **T**, *MYCN* continues to be expressed in the developing pituitary, unlike in the ventricular myocardium (**V** *vs* **W**).

Abbreviations: ad: adrenal gland, aer: apical ectodermal ridge, ao: aorta, br: bronchi, di: diencephalon, drg: dorsal root ganglia, eg: enteric ganglia, g:gut, g/IX/X: cranial ganglia IX/X, gt: genital tubercule, h: heart, hg: hindgut, inf: infundibulum, lb: limb bub, lv: liver, mes: mesencephalon, mx: maxilla, nt: neural tube, oe: oesophagus, rb: rathke's pouch, rh:

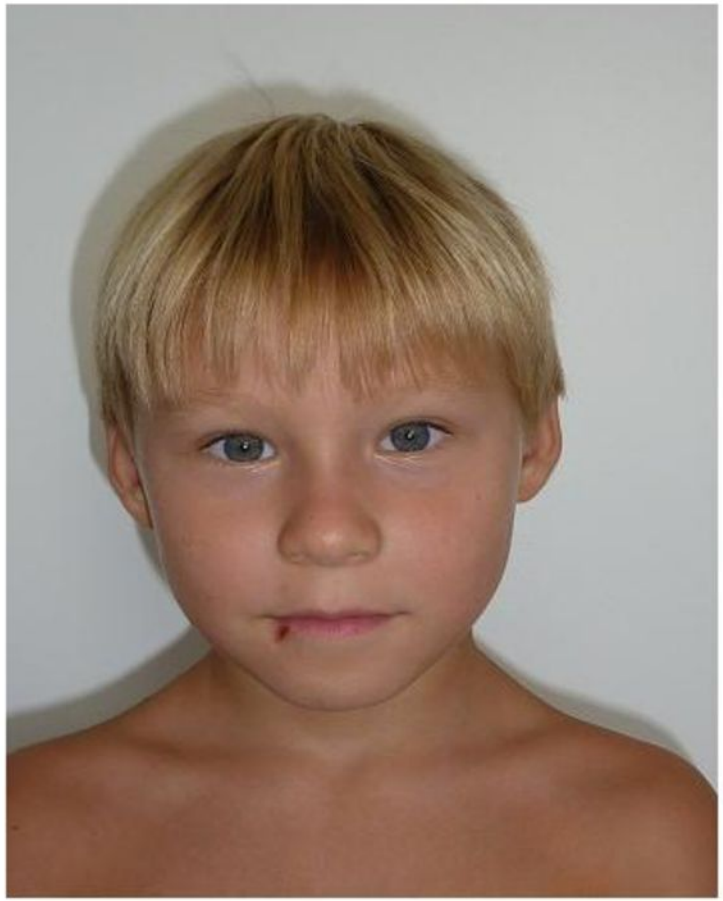
rhombencephalon, sc: spinal cord, sg: sympathetic ganglion, st: stomach, tel: telencephalon, tg: tongue, umb: umbilical cord.

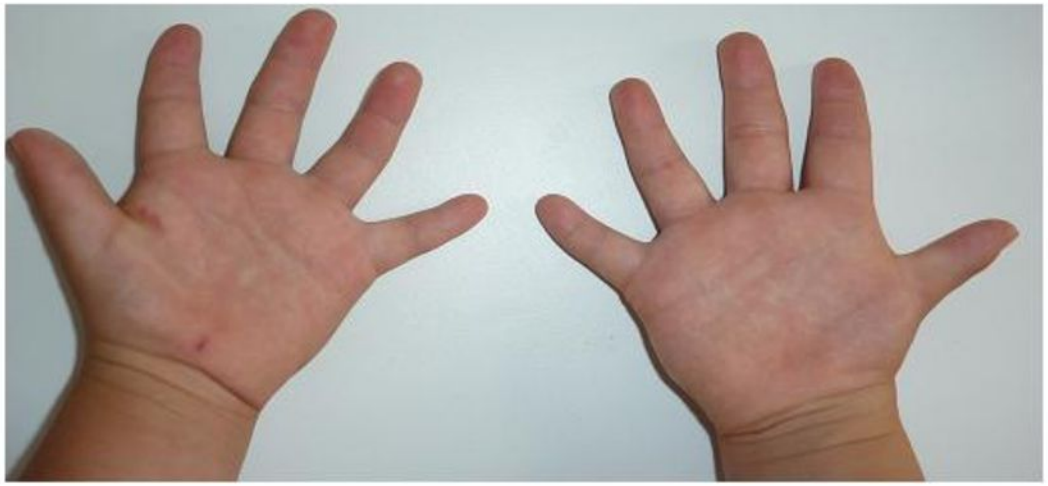
Scale bars: 0.5 mm except for **Q**-**S**, 1mm.

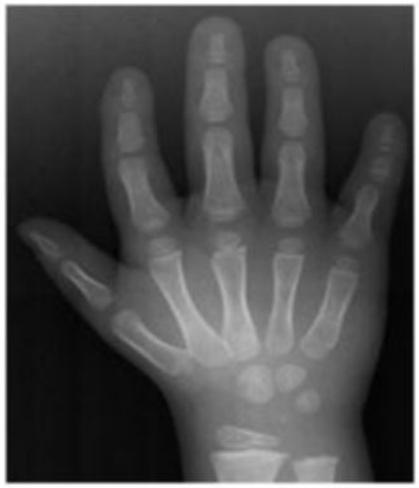
Mutated Patients	AO2	AO28	AO37	A056	A060	A065	A067	A068	Total
Sex	F	F	M	M	F	M	F	M	4M/4F
Familial history	-	+	-	-	+	-	+	-	3/8
Head circonference at birth	–2	-3	-4	-3	-2	-4	-3	-2	8/8
Post natal microcephaly (SD)	–3	-3	-4	-3	-2	-4	-3	-2	8/8
Weight and size at birth	50thc.	25thc.	25 to 50thc.	25 to 50thc.	25 to 50thc.	25 to 50thc.	25 to 50thc.	25 to 50thc.	
Post natal growth retardation (SD)	-2	-2.5	-2	-2	-1	-2	0	0	5/8
Mental retardation	Mild	Moderate	Mild	Mild	Mild	Moderate	Mild	Mild	8/8
Micrognatia	-	-	-	-	-	+	+	-	2/8
Brachymesophalangy II et V	+	+	+	+	+	+	+	+	8/8
Toe Syndactyly 2/3	-	+	-	+	+	+	+	-	5/8
Toe Syndactyly 4/5	+	+	+	-	-	+	+	-	5/8
Oesophageal atresia	+	+	+	+	+	+	+	-	7/8
Duodenal atresia	-	-	-	-	-	-	-	-	0/8
Renal hypoplasia	+	-	+	-	-	-	-	-	2/8
Congenital cardiac defect	ASD	VSD	-	-	-	-	-	-	2/8
Deafness	-	-	-	-	-	-	-	-	0/8
Asplenia	-	+	-	-	-	-	-	-	1/8
Result of *MYCN* gene sreening	c.1180G>A	c.1293delC	c.1110insG	c.928-930insGT	c.474-514del	c.1177C>T	c.134dupC	del 2p24.3	8/8

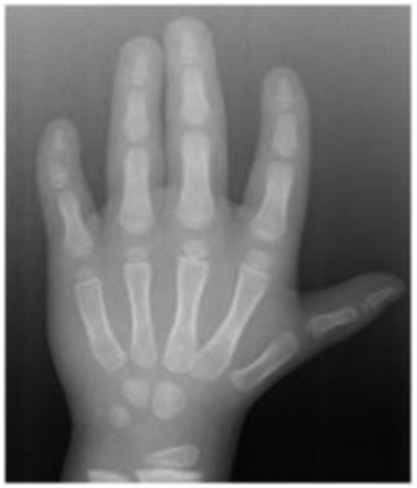
Non Mutated Patients	AO3	AO4	AO22	AO35	AO36	AO39	AO41	AO42	AO43	Total
Sex	M	F	F	M	M	F	M	F	F	4M/5F
Familial history	-	-	-	-	-	+*	+**	-	-	2/9
Head circonference at birth	–2.5	0	-2	0	-2.5	0	–3	-4	-1	5/9
Post natal microcephaly (SD)	–2.5	-2	-2.5	-2	-2.5	–3	–3	-4	-2	9/9
Weight and size at birth	25 to 50thc.	50thc.	50thc.	50thc.	50thc.	50thc.	25 to 50thc.	50thc.	50thc.	
Post natal growth retardation (SD)	-1	-1	-	-2	-1.5	0	-2.5	-3	0	3/9
Mental retardation	Mild	Moderate	Mild	Mild	Mild	Mild	Moderate	Mild	Mild	9/9
Micrognatia	-	+	-	+	-	-	-	-	-	2/9
Brachymesophalangy II et V	+	-	+	+	+	+	+	+	-	7/9
Toe Syndactyly 2/3	-	+	+	-	-	+	-	+	-	4/9
Toe Syndactyly 4/5	-	-	-	+	-	+	-	-	-	2/9
Oesophageal atresia	+	+	-	+	+	+	-	+	+	7/9
Duodenal atresia	-	-	-	+	-	-	-	-	-	1/9
Renal hypoplasia	-	-	-	-	-	-	-	-	-	0/9
Congenital cardiac defect	-	-	-	-	VSD, MA, AC	-	-	-	VSD	1/9
Deafness	-	-	-	+	-	-	-	-	-	0/9
Asplenia	-	-	-	-	-	-	-	-	-	0/9
Result of *MYCN* gene sreening	-	-	-	-	-	-	-	-	-	0/9
Result of Nimblegen fine-tiling array	normal	normal	normal	normal	normal	normal	NP	NP	NP	0/6
Result of 244K genome wide array	normal	normal	normal	normal	normal	NP	NP	NP	NP	0/5

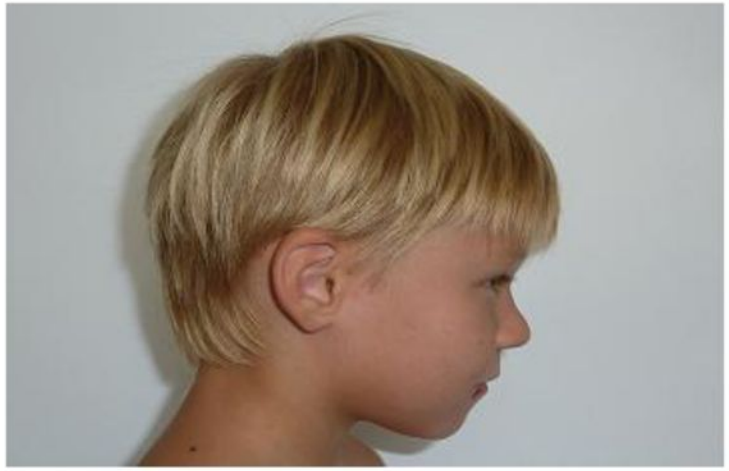
Table 1

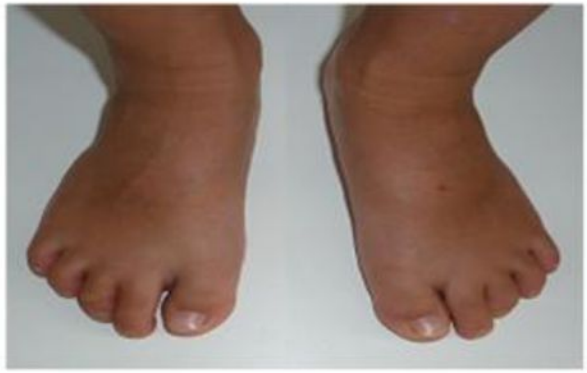


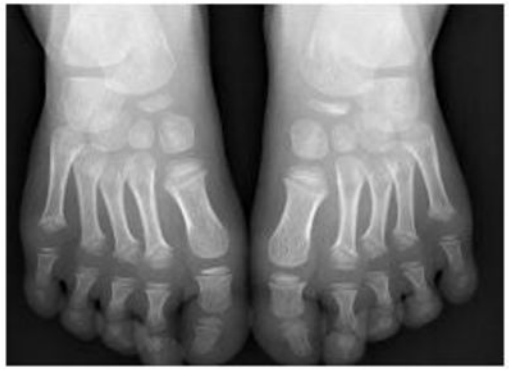












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c.928-930insGT; p.Pro310ValfsX9

c.1110insG p.Lys368fsX

c.1177C>T; p.Arg393Cys

c.1180G>A; p.Arg393His

c.1293delC; p.S432fs

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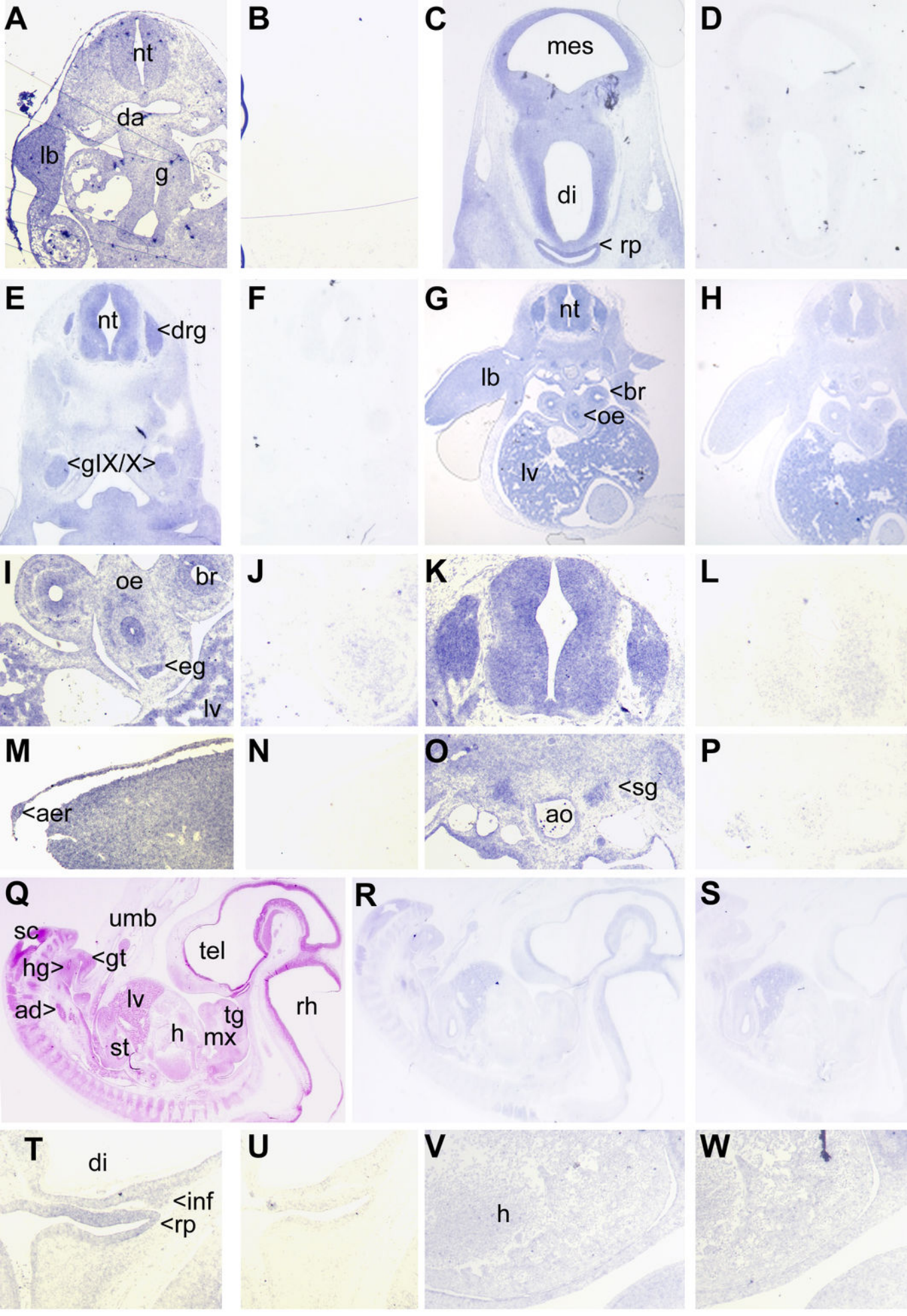
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