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JAG1 loss-of-function variations as a novel predisposing event in the pathogenesis of congenital thyroid defects

--Manuscript Draft--

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Abstract:	Context: The pathogenesis of congenital hypothyroidism (CH) is still largely unexplained. We previously reported that perturbations of the Notch pathway and knockdown of the ligand jagged1 cause a hypothyroid phenotype in the zebrafish. Heterozygous JAG1 variants are known to account for Alagille syndrome type 1 (ALGS1), a rare multisystemic developmental disorder characterized by variable expressivity and penetrance. Objective: Verify the involvement of JAG1 variants in the pathogenesis of congenital thyroid defects and the frequency of unexplained hypothyroidism in a series of ALGS1 patients. Design, Settings, Patients: A total of 21 young ALGS1 and 100 CH unrelated patients

	were recruited in academic and public hospitals. The JAG1 variants were studied in vitro and in the zebrafish. Results: We report a previously unknown non-autoimmune hypothyroidism in 6/21 ALGS1 patients, 2 of them with thyroid hypoplasia. We found two JAG1 variants in the heterozygous state in 4/100 CH cases (3 with thyroid dysgenesis, 2 with cardiac malformations). Five out 7 JAG1 variants are new. Different bioassays demonstrate that the identified variants exhibit a variable loss-of-function. In zebrafish, the knock-down of jag1a/b expression causes a primary thyroid defect, and rescue experiments of the hypothyroid phenotype with wild-type or variant JAG1 transcripts support a role for JAG1 variations in the pathogenesis of the hypothyroid phenotype seen in CH and ALGS1 patients. Conclusions: clinical and experimental data indicate that ALGS1 patients have an increased risk of non-autoimmune hypothyroidism, and that variations in JAG1 gene can contribute to the pathogenesis of variable congenital thyroid defects, including CH.
Secondary Abstract:	Patients with Alagille syndrome have an increased risk of non-autoimmune hypothyroidism and JAG1 gene variants can contribute to the pathogenesis of congenital thyroid defects.
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Milan, December 10th, 2015

Dear Dr Robertson,

hereby, I kindly ask you to evaluate the revised version of our manuscript JC-15-3403 for publication in JCEM as an Original Article.

The title of the manuscript is "JAG1 loss-of-function variations as a novel predisposing event in the pathogenesis of congenital thyroid defects".

The authors of the manuscript are: Tiziana de Filippis, Federica Marelli, Gabriella Nebbia, Patrizia Porazzi, Sabrina Corbetta, Laura Fugazzola, Roberto Gastaldi, Maria Cristina Vigone, Roberta Biffanti, Daniela Frizziero, Luana Mandarà, Paolo Prontera, Mariacarolina Salerno, Mohamad Maghnie, Natascia Tiso, Giorgio Radetti, Giovanna Weber and Luca Persani.

We were delighted to read the positive comments of the Reviewers. In this revision, we made a series of novel experiments that were asked by the Reviewer 1 in order to demonstrate the specificity and improve the quality of our jag1a/b gene knock-down in the zebrafish. We believe that we addressed all the comments raised and provide enclosed a rebuttal illustrating all the changes that were made into our manuscript. We also included a structured abstract. The revised text was highlighted in yellow all along the revised manuscript. We believe that the manuscript has significantly improved and provides data supporting JAG1 gene variations as a novel predisposing factor in the pathogenesis of thyroid defects, including congenital hypothyrodism.

All the Authors approved the revised version of this manuscript. On behalf of all Authors, I declare no conflict interest related to this work.

I hope that you will find our revised manuscript worthy for consideration, and look forward your decision.

Sincerely yours,

Luca Persani, MD, PhD
Corresponding Author

Reviewers' comments:

Reviewer #1: Filippis and colleagues describe the identification of alterations in Jagged1 as a predisposing event in the pathogenesis of congenital thyroid defects. Building off their prior work identifying a role for jag1a and jag1b in thyroid development in zebrafish, they analyze the role of Jagged1 in the pathogenesis of congenital thyroid defects in human disease. They find an association between ALGS1 patients and non-autoimmune hypothyroidism. They identify features of non-autoimmune mild hypothyroidism in 6/21 ALGS patients, and in 2/6 of these patients thyroid hypoplasia. The authors identify two variants in JAG1 in 4/100 patients with congenital hypothyroidism. They perform subcellular localization of JAG1 variants in NIH-3T3 cells by overexpression and immunofluorescence. They test the variants in a functional assay for differentiation of C2C12 myoblasts.

The authors use a functional assay in zebrafish to determine the consequence of expressing JAG1 variants. The authors perform morpholino based knockdown, using previously published morpholinos targeting jag1a and jag1b and determine the consequence on global developmental morphology and thyroid differentiation markers. This is not terribly novel in light of their prior published work (Porazzi, Endocrinology 2012), though they do look at combined knockdown in this study and look at markers by confocal, which is new.

R.: The experiments here performed show for the first time the results of the combined knock-down of both jag1a/b genes in zebrafish. In addition to the determination of thyroid markers we also show for the first time the results of an increased tshba expression after jag1a/b silencing, a figure that definitely confirm the generation of a primary thyroid defect.

The authors compare the efficiency of wild-type human JAG1 and variants they identify in a zebrafish based rescue assay. This critical data is not totally convincing to me. In Fig 4 panel A, the histogram labeling and figure legend is unclear. What exactly was injected?

R.: We thank the reviewer for highlighting this. We made more clear the legend of the figure and we clarified the different experimental conditions under the X axis.

If it is each variant alone then the '+' is misleading. In Fig. 4 panel B the ability of wild-type vs. variant JAG1 isoforms is compared. Why are such large numbers of embryos needed to make this point?

R. The advantage of this model animal is the possibility to evaluate a large number of samples at the same time. This allows the possibility to confer a significant impact to variations that in other models or assays are hard or more expensive to obtain.

This would be more convincing if the authors showed representative examples and specific markers. The authors perform a reporter assay using an NICD reporter in zebrafish. This demonstrates functional differences between Jag1 variants in an in vivo reporter gene assay in zebrafish.

Major points:

Recently significant issues related to morpholino specificity have been highlighted as a major concern in zebrafish genetic studies (Kok, et al Developmental Cell Jan 2015). Many investigators are shifting to genome editing using CrispR/Cas9 for this reason. In this manuscript the authors use previously tested morpholinos targeting jag1a/1b. My main concern is that the evidence that these reagents rigorously

knockdown jag1a/1b expression may not be as strong as desirable. The minimum data needed to validate these tools would include:

R: We thank the reviewer for this comment. Indeed, we are aware of the risk associated with the use of morpholinos, as with other different genetic manipulations. We indeed, verified that our morpholinos induced specific knock-down effects. Of note, in the original paper on the perturbation of Notch pathway and consequent effects on thyroid development in zebrafish (Porazzi et al. "Disruption of global and JAG1-mediated Notch signaling affect thyroid morphogenesis in the zebrafish" that was published In Endocrinology 2012; ref 6 in this manuscript) we tested by RT-qPCR the effects obtained on jag1a and jag1b by morpholino knock-down, and in the case of jag1b also by analysis of the zebrafish jag1b^{b1105} mutant line. The thyroid phenotype was indeed similar in jag1b morphants and mutants confirming the specificity of jag1b knock-down.

1. Demonstrate targeting of the splice junction by RT-qPCR for each morpholino separately.

R1. This issue was previously investigated in the reference 6. In the supplemental Figure 4 of re. 6, we showed the specific morpholino-mediated knock-down of jag1a and jag1b, separately. The Legend of Figure 2 was revised and includes this information.

2. Demonstrate that combining the morpholinos targets both genes by qPCR

R2. The effects of the combined knock-down on jag1a and jag1b expression (by semi-quantitative and quantitative RT-PCR) by means of the co-injection of both morpholinos are now shown in Figure 2, panels B, C, D. The efficiency of the combined knock-down was similar to that seen for each morpholino separately (see supplemental fig 4 of ref 6).

3. Explore whether there is an antibody that can be used to measure jag1a/b levels in zebrafish.

R3. Antibodies against Jag1 are indeed available but made against human or mouse jag1 proteins. We indeed had tested several of these antibodies but the results were quite deceiving as none of them gave reliable results in the zebrafish.

4. Provide higher quality images of JAG1 rescued morphants.

R4: We thank the reviewer for this criticism. We provide a revised Figure 4, that includes new panels with images of improved quality and illustrating the rescue phenotypes in more details. The legend of Figure 4 was also revised accordingly.

5. Include an analysis of thyroid marker genes by whole mount in situ after JAG1 rescue.

R5: The revised Figure 4 now includes panels G-L that show the thyroglobulin expression (lateral and ventral views) in the control fish and in the rescued vs non-rescued zebrafish morphants. Note that rescued morphants exhibit a normal thyroid morphology and volume at 72 hrs.

6. The authors bin the morpholino phenotypes into (3) classes which correlate nicely with thyroid marker expression. They need to correlate these phenotypes with the efficiency of morpholino knockdown using a molecular assay.
R6. We thank the reviewer for the suggestion. In the revised figure 2 (panels C, D) we correlated the observed phenotype classes with the efficiency of jag1a/b knock-down. Indeed, the severity degree of the phenotype was correlated with a more profound knock-down of jag1a/b. Indeed, a more profound knock-down of jag1b was associated with an early lethality (<24 hrs).

Minor Points

1. Fig 4A histogram and legend is unclear. See above.
R. This figure and legend were completely revised, accordingly (see R4 and R5)

Reviewer #2: The authors investigated the role of JAG1 in congenital hypothyroidism. Assisted by several institutions within Italy, 100 unrelated subjects with congenital hypothyroidism were investigated as well as 21 patients with ALGS1 syndrome. Six of the 21 individuals with ALGS1 were found to have various degrees of hypothyroidism and of the 100 patients with congenital hypothyroidism, 4 were found to have a variant JAG1.

CRITIQUE

The authors provide a detailed study of their cohort, both in terms of clinical information as well as in vitro and in zebra fish, studies to better characterize the putative pathogenicity of these variants.

The studies were well conducted. It is interesting that inheritance was paternal in 4 cases and maternal in 2. However, the samples are too small to draw conclusions.

R: We thank the Reviewer for the positive comments. Indeed, we cannot draw conclusion on sex-related pattern of inheritance due to the limited size of the sample. However, these data provide clues against the possible involvement of parental imprinting.

The authors have a tendency to dramatize the effect of the JAG1 variants. More specifically, in the summary of in silico prediction of the JAG1 variants effect (Supplemental Table 2), R744Q is listed as "damaging" for SIFT and "possibly damaging" for polyphen2 when the scores in ensembl are 0.06 and 0.086, respectively, both tolerated.

R: We thank the Reviewer for this information. Due to the awareness on the limited value of the in silico prediction, we indeed tested the missense/truncated/frameshift variants by means of 7 different algorithms and, in order to avoid bias due the choice of one specific prediction algorithm, we gave a summary of the deleterious predictions in the supplemental Table 2.

Of note, we indeed interrogated the different algorithms, including SIFT and PolyPhen-2, through an updated professional software (Alamut Visual 2.4) and not through the public available version of these two algorithms on ENSEMBL. Different versions of the prediction algorithms may explain such discrepancy. In fact, on the website of ENSEMBL it is clearly stated that: "Different versions of the protein databases can

result in substantial variance in the predictions and scores obtained. ENSEMBL releases are not synchronized with updates to the SIFT and PolyPhen webservers, so differences are to be expected". This is indeed another argument supporting the use of various algorithms for the prediction on the damaging effect of one gene variant.

JAG1 loss-of-function variations as a novel predisposing event in the pathogenesis of congenital thyroid defects

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Conflict of interest: The Authors have nothing to disclose.

Abstract

Context: The pathogenesis of congenital hypothyroidism (CH) is still largely unexplained. We previously reported that perturbations of the Notch pathway and knockdown of the ligand jagged1 cause a hypothyroid phenotype in the zebrafish. Heterozygous *JAG1* variants are known to account for Alagille syndrome type 1 (ALGS1), a rare multisystemic developmental disorder characterized by variable expressivity and penetrance.

Objective: Verify the involvement of *JAG1* variants in the pathogenesis of congenital thyroid defects and the frequency of unexplained hypothyroidism in a series of ALGS1 patients.

Design, Settings, Patients: A total of 21 young ALGS1 and 100 CH unrelated patients were recruited in academic and public hospitals. The *JAG1* variants were studied in vitro and in the zebrafish.

Results: We report a previously unknown non-autoimmune hypothyroidism in 6/21 ALGS1 patients, 2 of them with thyroid hypoplasia. We found two *JAG1* variants in the heterozygous state in 4/100 CH cases (3 with thyroid dysgenesis, 2 with cardiac malformations). Five out 7 *JAG1* variants are new. Different bioassays demonstrate that the identified variants exhibit a variable loss-of-function. In zebrafish, the knock-down of *jag1a/b* expression causes a primary thyroid defect, and rescue experiments of the hypothyroid phenotype with wild-type or variant *JAG1* transcripts support a role for *JAG1* variations in the pathogenesis of the hypothyroid phenotype seen in CH and ALGS1 patients.

Conclusions: clinical and experimental data indicate that ALGS1 patients have an increased risk of non-autoimmune hypothyroidism, and that variations in *JAG1* gene can contribute to the pathogenesis of variable congenital thyroid defects, including CH.

Introduction

Congenital hypothyroidism (CH) is the most common endocrine developmental disorder and preventable cause of mental retardation. In recent years, several groups from various socio-economically developed countries described a tendency toward an increased CH incidence (about 1 out 1500-2000 newborns) (1-3). Both functional and developmental defects can account for CH but its etiology is still poorly understood (4,5). Genetic defects are believed to be frequently involved, but the disease is generally reported to be sporadic and variations in candidate genes have been found in <10% of the cases (4).

A recent work from our group in the zebrafish model (6) demonstrated a role for Notch pathway in thyroid morphogenesis and function. Notch pathway is a highly conserved cell-to-cell communication system involved in the specification of many tissues, and its alterations have been involved in several developmental diseases (7). Several ligands, including Jagged proteins, expressed on the cell surface interact with the Notch receptors of adjacent cells. This interaction results in cleavage and subsequent translocation of the Notch receptor intracellular domain (NICD) into the nucleus, where it interacts with a variety of transcription factors and repressors to modulate the expression of specific genes (8). In particular, we found that zebrafish *jag1a* and *jag1b,* orthologous of the human *JAG1* encoding one single-pass trans-membrane ligand of the Notch receptors, are expressed in the developing thyroid and individually contribute to its growth and function (6). Heterozygous *JAG1* variations are known to cause Alagille syndrome type 1 (ALGS1), a multisystemic congenital disorder characterized by a highly variable involvement of liver, heart, skeleton, eye and facial defects (9). Intriguingly, both CH and ALGS1 are characterized by variable expressivity and penetrance even within familial cases (10,11).

In this work, we investigated whether *JAG1* gene variants might be involved in inborn thyroid defects. Therefore, we analyzed the thyroid function in a series of young ALGS1 patients carrying heterozygous *JAG1* variants, and screened *JAG1* coding sequence in patients with CH. The identified *JAG1* variants have been functionally tested in a series of bioassays in cellular and

zebrafish models. The results here reported indicate non-autoimmune hypothyroidism as a recurrent finding among ALGS1 patients, and *JAG1* loss-of-function (LOF) variations as an novel predisposing cause in the pathogenesis of congenital hypothyroidism.

Materials and Methods

Patients

Two groups of patients were enrolled in this study:

- 21 unrelated ALGS1 patients carrying non-synonymous variations in *JAG1* gene that had been referred to our center for genetic diagnosis of Alagille syndrome;
- 100 unrelated CH subjects (66 thyroid dysgenesis and 34 gland-in-situ).

The thyroid imaging was performed with ultrasound and/or scintigraphy. The thyroid volume (TV) on ultrasound was calculated by the ellipsoid formula, as previously done (12).

Genetic investigation was extended to a control group of 349 blood donors. Institutional ethical committees approved the study, and informed consent for blood sampling and genetic investigations were obtained from all participants or from parents.

Experimental procedures

Details on the genetic screening, bioinformatics analyses, cloning, mutagenesis and transfection of *JAG1* constructs, as wells as protocols of the functional assays in vitro (cellular expression and evaluation of the differentiation potential of *JAG1* variants) or in vivo in the zebrafish model (knock-down of *JAG1* orthologues with morpholino antisense oligos and characterization of jag1-deficient fish, rescue experiments of the phenotype of jag1-deficient fish using human *JAG1* variants and Notch-dependent luciferase assay) are reported in the Supplemental material. Fish were maintained and raised according to the EU (Directive 2010/63/EU) and Italian (Decreto legislativo 116/92) regulations on laboratory animals, and the study was approved by the institutional ethical committee.

Statistical Analysis

Statistical analyses were carried out using the software package PRISM 4.0 (GraphPad, San Diego, CA). Results are expressed as mean ± SE. Kruskal-Wallis test followed by Dunn's Multiple Comparison Test were used. All data were shown as means ± standard error (SEM) or percentages. The Mann-Whitney test and the *t*-student test were used to comparison of differences between groups. The $p<0.05$ was considered statistically significant (*$p<0.05$; ** $p<0.01$; ***$p<0.001$). All the analyses were conducted with the software package GraphPad Prism 4.0 (GraphPad, San Diego, CA).

Results

Thyroid function in ALGS1

The biochemical and ultrasound investigations revealed that 6 out 21 ALGS1 patients (28.6%) have the biochemical features of non-autoimmune mild hypothyroidism (high TSH and fT4 levels in the lower part of the normal range). The thyroid imaging revealed a thyroid hypoplasia in 2 of these ALGS1 cases (Table 1). All patients had non-synonymous *JAG1* variations and the clinical and genetic features typical of ALGS1, variably including chronic liver failure, congenital heart defects, facial dysmorphism, skeletal abnormalities or embriotoxon (Table 1). Inheritance of *JAG1* variants could be assessed in all cases but one: the variant found in ALGS_4 occurred *de novo,* whereas the maternal or paternal inheritance was seen in the others. In the families, the phenotypical expression of the ALGS1 defects was highly variable among the carriers (Table 1).

Genetic screening of JAG1 gene in CH patients

Among 100 CH patients, we identified two distinct *JAG1* sequence variants in four unrelated patients. The first missense variation, p.R937Q (rs145895196, minor allele frequency, MAF: 0.0022), already described in different ALGS cases (13,14) was found in 3 cases. The patient identified by CH1 was affected by apparent athyreosis and an atrial septal defect; the patients CH3 and CH4 had an apparent athyreosis or a gland-in-situ, respectively, associated with no other

evident abnormality (Table 1). The second variant, p.R744Q (rs147809756, MAF: 0.0010) (15), was found in patient CH2 with thyroid ectopy, pulmonary artery atresia and ventricular septal defect. In all these cases, we excluded the presence of other mutations in known candidate genes (*NKX2-1*, *FOXE1*, *PAX8*, *NKX2-5*, *TBX1* and *SHH*). Furthermore, none of these four patients showed clinical features consistent with ALGS1. In two cases, the inheritance of the variant could be assessed. The mothers of CH2 and CH4 had also non-autoimmune mild hypothyroidism (Table 1).

Bioinformatics analyses

We identified seven different *JAG1* variants, four missense changes affecting residues that are well conserved across species (Supplemental Fig 1), two causing a premature truncation of the translated protein, and one deletion affecting the donor splice site after exon 18. All variations affect functionally relevant protein domains (Supplemental Table 1). Five variations are new, whereas two had been previously reported in ALGS1 patients (13-15) and in 1000 Genomes database, and were also found in 3 out of 349 subjects of the control population (0.0086%; P=0.046 vs CH).

The functional impact of the missense or truncated variants was tested by 7 online programs. The new variants p.T587I, p.C917G, p.N1026EfsX8 and p.I1035X are invariably predicted to be potentially damaging (Supplemental Table 2). The rare variants p.R744Q and the p.R937Q are predicted to be deleterious by 4 or 2 out of 7 algorithms, respectively. All these variants had no potential splicing effects, in contrast with the intronic deletion (IVS_18 c.2860+1 delAG) (Supplemental Table 3).

Immunofluorescence localization of JAG1 variants

The Jagged1 sub-localization was studied in transiently transfected NIH-3T3 cells by confocal microscopy. The immunodetection of Jagged1 ligand revealed that the wild-type protein is largely expressed on the cell membrane, and only a minor component is detectable in intracytoplasmatic compartments (Fig. 1A). The missense variants p.T587I, p.R744Q and p.R937Q are also efficiently expressed on the cell membrane, in contrast with truncated proteins (p.N1026E*fs*X8 and p.I1035X)

and the missense variant p.C917G, which are almost completely retained in the perinuclear region (Fig. 1A). The amount of Jagged1 protein expression was similar for all constructs (data not shown).

In vitro differentiation assay

The ability of Jagged1 ligand to affect the terminal differentiation of the C2C12 myoblasts is well documented (16,17). The C2C12 myoblasts cell line has been widely used as a model system of myogenesis, since the differentiation program of these cells is readily induced, easily monitored, and highly reproducible (18). When C2C12 cells are cultured in mitogen-poor medium, they differentiate into multinucleated myotubes and express muscle lineage-specific genes, such as *MyoD* and *MRF4* (19). This differentiation program is inhibited in the presence of functional Jagged1 ligands and, indeed, the expression of these muscle differentiation genes is strongly repressed after transfection of wild-type *JAG1* (Fig. 1B). At variance, the transfection of mutant constructs does not significantly affect the expression levels of *MyoD* and *MRF4* genes, though a non significant tendency toward a reduced expression of the differentiation markers can be observed after transfection of p.T587I, p.R744Q and p.R937Q constructs. These results are therefore consistent with a loss of function (LOF) of *JAG1* variants.

Evaluation of the phenotype in jag1_MOs injected zebrafish embryos

In order to study the *in vivo* effects of *JAG1* variants, we took advantage of the vertebrate zebrafish model. After 24 hpf of the co-injection of jag1a and jag1b mopholinos (jag1_MO), thousands of embryos were compared with mismatch controls (mism_ctrls) and classified into three graded phenotype classes, depending on the severity of defects in the body length and curvature. Class 1 (C1_MO): mild phenotype: moderately shortened body. Class 2 (C2_MO): intermediate phenotype: shortened body and notochord abnormalities. Class 3 (C3_MO): severe phenotype: shortened body, notocord defects and twisted or truncated tail (Fig. 2A). The phenotypic classification correlates with the diminished expression of wild-type (long) *jag1a* and *jag1b* and the specular increase of aberrant (short) trancripts induced by morpholino injection (Fig. 2B-D). We performed ten

192 independent experiments of jag1_MO injection and distributed the embryos in the three classes with
193 the following frequency: C1_MO: 22%; C2_MO: 63%; C3_MO: 15%. Of note, all jag1_MO
194 embryos dysplay craniofacial abnormalities such as small head and eyes, thin midbrain-hindbrain
195 boundary and, in 30% of cases, cardiac edema.
196 The mism_ctrls exhibit a normal expression of early (*nkx2.4b*) and late (*slc5a5* and *tg*) thyroid
197 markers (Fig.2, panels E, I, N). Of note, the thyroid phenotype was highly homogeneous among
198 each group (around the 90-95% of embryos) and well correlated with the severity of the defects
199 found in jag1_MOs. At 24 hpf, the expression of *nkx2.4b* in the thyroid primordium was slightly
200 reduced in the C1_MO embryos when compared with mism_ctrls (Fig. 2F), and at 48 and 72 hpf,
201 the C1_MO embryos exhibited a negligible decrease of *tg* and *slc5a5* staining whilst mantaining a
202 normal thyroid shape (Fig. 2, panels J, O). In C2_MOs, a significant reduction of *nkx2.4b*
203 expression at 24 hpf (Fig. 2G) is evident; the impared thyroid primordium specification is followed
204 by a diminished expression of *tg* and *slc5a5* at 48 and 72 hpf, and a decreased thyroid size lacking
205 the typical elongation of the zebrafish thyroid along the ventral aorta at 72 hpf (Fig. 2, panels K, P).
206 In C3_MOs, the thyroid primordium is absent or severly reduced all along the period of observation
207 (Fig. 2, panels H, L, Q).
208 To understand the level of thyroid function impairment in the different classes of jag1_MOs, we
209 concomitantly quantified the expression *tshba* and *tg* by confocal microscopy of embryos subjected
210 to *in situ* hybridization and qRT-PCR (Fig. 3). The different grades of the hypothyroid phenotype
211 exhibited by jag1_MOs is associated with a corresponding increase in the expression of *tshba* gene,
212 consistent with a primary defect of the thyroid gland. In particular, in the C2_MOs the reduced *tg*
213 expression was associated with a significant increase in the expression of *tshba* at both 48 hpf (Fig.
214 3, panels C and C' and I) and at 72 hpf (Fig. 3, G and G' and K). The severe impairment of thyroid
215 function in C3_MOs induced a further increase of *tshba* expression in the thyreotrope cells at 48
216 hpf (Fig. 3, D and D' and I) and at 72 hpf (Fig. 3, H and H' and M).
217 After confocal evaluation, forty embryos from each pool were used for qRT-PCR analysis (Fig 3, J

and L). These data confirmed those obtained by confocal microscopy at 48 and 72 hpf.

Rescue of thyroid function with hJAG1 constructs

In order to evaluate the effects of the h*JAG1* variants on thyroid function *in vivo*, we co-injected the purified human transcripts with the morpholinos against the zebrafish *jag1a* and *jag1b*. After five indipendent experiments, we splitted the thousands of embryos into two major groups: group 1 (G1, rescued morphological phenotype and normal *tg* expression): correct body length, null/slight notochord and craniofacial defects and normal thyroid phenotype; group 2 (G2, unreverted phenotype/ hypothyroidism as seen in C2_ and C3_MOs): reduced body length, moderate/severe notochord and craniofacial defects and reduced size of *tg* expression (Fig. 4, Panels A-L). The volume of tg positive cells was similar in G1 or G2 classes, through all conditions (Fig. 4N).

The injection of human *JAG1*_WT transcript is associated with a normal phenotype and thyroid function in 75% of the cases, resulting in a rescue efficiency of 55% (percent increase of G1 embryos over jag1_MOs); these embryos dysplay a significant improvement of *tg* expression reaching values similar to those of the mism_ctrls (Fig. 4M). The co-injection of missense p.T587I, p.R744Q and p.R937Q transcripts are able to significantly revert the morphological and hypothyroid phenotype (+29%, +35% and + 25% of G1 embryos, respectively), but these rescue efficiencies were significantly lower than that of wild-type trascript. Finally, the injection of the p.C917G mRNA leads to a rescue efficiency of only 13% (p=NS vs jag1_MOs), while the truncated forms (p.N1026EfsX8 and p.I1035X) always failed to revert the jag1_MO phenotype (Fig. 4M).

In vivo zebrafish luciferase assay

An *in vivo* dual-luciferase assay was used to investigate the ability of the *JAG1* variants to interact with Notch receptors and activate the downstream intracellular signals. The zebrafish embryos were co-injected with a luciferase reporter gene construct (pGa981-6-Firefly), containing the hexamerized NICD-responsive RBP-Jk binding motif, and growing amount of different *JAG1* constructs. We co-injected the same amount of the pGa981-6-Firefly in the presence of growing concentrations (2.5-50 pg/embryo) of the pcDNA3.2/V5-DEST *JAG1*_WT. The activity of the

Firefly luciferase is strictly dependent upon the amount of the *JAG1*_WT injected reaching the plateau at 40-50 pg/embryo, and the concentration of 25 pg/embryo was chosen to analyze the *JAG1* variants (Fig. 5A). In agreement with the rescue experiment, the missense *JAG1* variants are only partially able to activate the NICD-responsive elements *in vivo*, with a significant impairment of luciferase activity induction when compared to the wild-type construct (p.R744Q: 70%; p.T587I and p.R937Q: 56%; p.C917G: 42% of *JAG1*_WT). The truncated constructs (p.N1026E*fs*X8 and p.I1035X) displayed a complete loss of the ability to enhance the expression of luciferase (Fig. 5, B).

Discussion

This is the first report that provides evidence for the association between Notch signaling defects and congenital thyroid disorders in humans.

In a cohort of young patients with clinical manifestations and genetic analyses consistent with Alagille syndrome type 1, we found biochemical data indicating a non-autoimmune hypothyroidism in 28% of the cases. In addition, we found that *JAG1* variants with partial LOF are significantly enriched among CH patients. In association with evidence obtained in the vertebrate model zebrafish, these data indicate the *JAG1* gene as an additional candidate involved in the pathogenesis of congenital thyroid defects.

Here, we describe 7 different *JAG1* LOF variants, 5 of which never reported. These variants were analyzed by several *in silico* prediction algoritms, and *in vitro* or *in vivo* bioassays. The missense variations fall in the EGF-like repeats or the cysteine rich (CR) domains that are relevant for Jagged1 function and interaction with Notch receptors. In particular, the p.C917G change may alter the protein conformation and is in fact associated with a poor membrane targeting, similar to that seen with the truncated variants. Differently, the intronic deletion at the donor site of intron 18 (IVS18 c.2860+1delAG) was not tested *in vitro*, but the *in silico* analysis performed with *ad hoc* predictive programmes indicates this deletion as highly deleterious. In addition, this variation co-segregated with the disease phenotype in the family ALGS_3. Consistent with the known

involvement of *JAG1* in the pathogenesis of ALGS, the heterozygous *JAG1* variations with a more severe or complete LOF are associated with ALGS1 phenotype, whereas CH is associated with two rare missense variants previously reported in ALGS1 (13-15) and here characterized by a partial LOF.

Since hypothyroidism has not been previously reported in Alagille syndrome, this could be explained by the severe clinical picture affecting most ALGS patients that may mask the clinical signs of a mild hypothyroid state. On the other hand, ALGS is also associated with manifestations (growth retardation in 87%, mental retardation in 2%) (20) that might indeed be worsened by untreated hypothyroidism. Our data therefore raise the need for evaluation of thyroid disorder in larger cohorts of ALGS1 patients because of an increased risk of thyroid dysfunction.

These clinical findings are consistent with data obtained in the zebrafish model (6). Here, we provide further evidence that the knock-down of jag1-notch signaling in zebrafish causes primary hypothyroidism due to a defective thyroid growth, and other developmental defects, that can be rescued by injection of human *JAG1* transcripts supporting the specificity of the morpholinos. Evidences obtained by characterizing thousands of fishes (Fig. 4) show that the rare variants associated with CH are defective in either the rescue experiments or *in vivo* luciferase bioassay, as well as in the *in vitro* studies. The different phenotypic classes described upon *jag1a*/*jag1b* inactivation could be the result of either stochastic biological events or unpreventable small variations in the amount of morpholinos contained in each injected drop, emphasizing the fine gene-dosage sensitivity of the developing embryos. The morphological defects of our jag1_MO embryos (craniofacial and notochord alterations and, infrequently, cardiac edema) are similar to the ones described in other reports (21-23). In particular, we observed cardiac defects (unlooped heart tube formation) in less than 30% of jag1_MO embryos from 72 to 120 hpf. We found that several steps of thyroid morphogenesis are affected in jag1_MOs: at 24 hpf the *nkx2.4b* expression is absent or significantly reduced, and in these latter cases the residual thyroid follicles fail to elongate along the ventral aorta at 48-72 hpf. The hypothyroid phenotype induced by a defective Jagged1-Notch signal

both in humans and zebrafish could at least partially result from a direct effect limiting the number of thyroid precursors reaching the differentiated state, as *jag1a* and *jag1b* had been previously reported to be expressed in the developing thyroid follicles (6), but a contribution of defective signals coming from the surrounding tissues (such as the developing heart or big vessels) is also plausible. Interestingly, 3 out of 4 CH cases with *JAG1* variations had a dysgenetic thyroid (apparent athyreosis/profound hypoplasia or ectopy) associated with congenital heart defects in two of them (in the absence of variations in other candidate genes for these complex CH phenotypes), suggesting a more likely involvement of *JAG1* variants in CH cases associated with thyroid dysgenesis and/or heart malformations.

Both ALGS1 and CH are diseases characterized by a variable expression and penetrance, consistent with the different phenotype recorded in the relatives of the probands, and confirming that still unknown mechanisms are likely to contribute to the clinical expression of *JAG1* gene defects. These mechanisms should however be multiple and different in ALGS1 versus CH, since *JAG1* variants with similar LOF can be seen in both diseases. Still undetermined alterations affecting the Notch signal or thyroid function are more likely to coexist with *JAG1* variations in ALGS1 or CH families, respectively. Consistently, homozygous *Jagged1* mutant mice died at E9.5-10.5 of widespread haemorrhage (24), while heterozygous *Jagged1* mutant mice developed mild ocular defects, and only the specific combinations of *jagged*/*notch* gene knockdowns in zebrafish or mice were able to perturb the biliary, kidney, pancreas and craniofacial development as in Alagille syndrome (22,24,25). Similarly, the study of double heterozygous *Pax8* and *Titf1* null mice (26) has recently confirmed the existence of modifier alleles and the importance of gene dosage in the pathogenesis of CH.

In conclusion, the whole of the clinical and experimental data indicate that ALGS1 patients have an increased risk of non-autoimmune hypothyroidism, and that heterozygous variations in *JAG1* gene represent a novel predisposing event contributing to the pathogenesis of congenital thyroid defects.

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323

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401

402 **Figure Legends**

403

404 **Figure 1. Panel A, sub-cellular localization of wild-type and mutant Jagged-1 proteins in**
405 **NIH3T3 cell lines.** Proteins were visualized by immunofluorescence with a monoclonal anti-
406 Jagged-1 antibody (1C4, Cell Signaling Technology) followed by Alexa Fluor 488 conjugated
407 secondary antibody (green signal). DNA was counterstained with DAPI (blue signal). Cell
408 membranes were labelled with Alexa Fluor 594-tagged wheat germ agglutinin (WGA, red). **Panel**
409 **B, Inhibition of muscles-specific gene expression by *JAG1* variants.** C2C12 cells were
410 transiently transfected with the different constructs. After incubation in DM for three days, total
411 RNA was extracted and the expression level of *MyoD* and *MRF4* were analysed by qRT-PCR.
412 Samples were normalized using the ß-actin expression as housekeeping gene. The ΔΔCT method
413 was used to calculate relative expression levels, Empty vector_DM sample was used as calibrator.
414 Statistically significant (*p< 0.05; ***p<0.0001) performed with Kruskal-Wallis test followed by
415 Dunn's Multiple Comparison Test.

416

417 **Figure 2. Panel A, Morphological classification of jag1_MO embryos.** Jag1_MO were analyzed
418 at 24 hpf and subdivided in three major classes, based on body length and curvature. From the left
419 to right: mismatch controls (mism_ctrls); Class 1 (C1_MO): mild phenotype: normal or slight
420 curved tail; Class 2 (C2_MO) intermediate phenotype: shortened body, curved tail and notochord
421 defects (arrowheads); Class 3 (C3_MO) severe phenotype: severely reduced body length, twisted
422 tail, notochord defects. In the bottom left: number of embryos for each phenotype. Lateral views,
423 anterior to the left. Scale bar: whole embryo 500 μm. **Panel B-D, Evaluation of *jag1a* and *jag1b***
424 **mRNA expression of injected embryos. Panel B:** *jag1a* and *jag1b* pre-mRNA and mRNA
425 structures resulted from co-injection of *jag1a* (E2i2) and *jag1b* (E3i3) morpholinos (*jag1*_MOs):
426 squared boxes indicate the exon, the black lines correspond to the introns. The position of the
427 primers used to analyze the altered splicing by RT-PCR or qRT-PCR is indicated by blue arrows.

Panel C: RT_PCR of *jag1a* and *jag1b* mRNAs in *jag1*_MOs. The 500 bp and 320 bp PCR products were obtained in the mism_ctrls for *jag1a* and *jag1b*, respectively; at variance, *jag1*_MOs injected embryos display a reduction of the wt form and the presence of shorter products in both cases. Interestingly, the phenotypic classes of *jag1*_MOs nicely correlate with the amount of the wt/short expressed transcript. The beta-actin gene was used as a reference gene. The sequence analysis of the shorted products reveals the partial skipping of the E2 into the *jag1a* and the complete E3 skipping into the *jag1b* mRNAs. **Panel D:** qRT-PCR of *jag1a* and *jag1b* mRNAs expression after morpholinos injection using different sets of primers (indicated by the blue arrows in Panel B) that discriminate between the wild-type and shorter products. Results are expressed as % between the absolute quantification of the wt or short PCR products of *jag1a* or *jag1b*. The *eef1a* was used as housekeeping gene. Similar transcriptional effects were obtained when *jag1a* and *jag1b* morpholinos had been injected separately (6). Consistently with the semi-quantitative RT-PCR, the phenotype of *jag1*_MOs correlates with the ratio of the wt/short mRNAs. **Panel E-Q, Evaluation of thyroid development on jag1_MO embryos.** Whole mount in situ hybridization showing *nkx2.4b* (corresponding to human *NKX2.1)*, *slc5a5* and *tg* transcripts in representative jag1_MOs and control fish at 24, 48 and 72 hpf. For each marker, 50 embryos of mism_ctrls and from each class of jag1_MOs were analyzed. Images in ventral view, anterior to the left. (**E, I** and **N**) mismatch controls (mism_ctrls) exhibit a normal expression of early (*nkx2.4b*) and late (*slc5a5* and *tg*) thyroid markers. (**F, G** and **O**) Class1 of jag1_MO (C1_MO): slight reduction of thyroid marker expression with normal thyroid differentiation at 48 and 72hpf. (**G, K** and **P**); Class2 of jag1_MO (C2_MO): significant reduction of *nkx2.4b*, *slc5a5* and *tg* expression, with a markedly reduced thyroid size at 72 hpf. (**H, L** and **Q**); Class3 of jag1_MO (C3_MO): severe reduction/undetectable levels of *nkx2.4b*, *slc5a5* and *tg*. Failure of thyroid primordium formation and thyrocyte differentiation at 72 hpf. Scale bar: (E-H) 50 μm, (I-Q) 20 μm.

Figure 3. Expression of *tshba* and *tg* in jag1_MOs. The induced expression of *tshba* (corresponding to human TSHβ) by the pituitary due to the defective thyroid gland in jag1_MOs, is consistent with a primary defect in jag1_MOs. Images of WISH/Fast Blue staining followed by confocal acquisition of *tshba* and *tg* in C1_MOs (**B** and **B'**, **F** and **F'**), C2_MOs (**C** and **C'**, **G** and **G'**) and C3_MOs (**D** and **D'**, **H** and **H'**) at 48 and 72 hpf, compared with mism_ctrls (**A** and **A'**, **E** and **E'**). Embryos are all flat mounted, in ventral view, with anterior to the top. Histograms **I** and **K** summarize results from quantification of total *tshba*- and *tg*-positive cell volume (mm^3) at 48 and 72 hpf, using confocal microscope analysis on the same region and number of sections for each embryo (20 embryos for each stage and phenotype). Graphs **J** and **L** show the *tshba* and *tg* expression analysed by qRT-PCR at 48 and 72 hpf, compared with mism_ctrls. Asterisks indicate statistically significant differences (Mann-Whitney test, *p<0.05 and **p<0.001).

Figure 4. Panel A-F, Phenotypic features of the rescued embryos coinjected with *jag1a* and *jag1b* morpholinos and the human JAG1 mRNAs. At 24 hpf, phenotype of mism_ctrls (**A** and **D**) in comparison to: group1_embryos (**B** and **E**) with reverted phenotype and normal/slight reduced body length, normal craniofacial development; group2_embryos (**C** and **F**) with not reverted phenotype and reduced body length/ curved tail, notochord and craniofacial defects. Lateral views, anterior to the left. Scale bar: whole embryo 500 μm, head 200 μm. **Panel H-L,** expression by WISH of *tg* in the G1 and G2 embryos and comparison with the mism_ctrls at 72 hpf. Images in lateral view (**G-I**) or in ventral view (**J-L**). Scale bar 200 μm and 50 μm, respectively. **Panel M**, histograms show the percentage of embryos inside the group 1 (G1) and group 2 (G2) at 24 hpf after injection of jag1_MO only, or jag1_MO plus 7.5 pg/embryo of human *JAG1*_WT or mutant mRNAs, as indicated. **Panel N,** consistently to the WISH experiment, the histograms show the normal *tg* expression in G1 and the defective *tg* expression in G2 embryos under the different conditions (injection as in Panel M). Results of *tg* volume (mm^3) are expressed as mean ± SD. *p<0.05 and **p<0.001, indicate statistically significant differences versus the jag1_MOs. #p<0.05

and ##p<0.001, indicate statistically significant differences between mutant and wild-type *JAG1*mRNAs.

Figure 5. Zebrafish luciferase assay of *JAG1*_WT and variant constructs. Panel A, Co-injection with the same amount of pGa981-6-Firefly plus pcDNA3.2/V5-DEST (empty) as negative control or *JAG1*_WT, at growing concentrations (2.5-50 pg/embryos). The relative luciferase activity (RLU) was calculated by normalization with the Renilla pRL-TK levels (Firefly/Renilla). **Panel B,** Co-injection of 25 pg/embryo of pGa981-6-Firefly with pcDNA3.2/V5-DEST or *JAG1*_WT and *JAG1*_variants. The luciferase activity (RLU) of h*JAG1* variants was compared to the wild type form. Asterisks indicate statistically significant differences versus *JAG1*_WT (Mann-Whitney test, *p<0.05 and **p<0.001).

Table 1. Clinical features of ALGS1 patients and CH infants with JAG1 variants.

Case Sex/Age*	***JAG1* variant**	**dbsTSH (mU/L)**	**Serum TSH (mU/L)**	**Serum FT4 (% of the lower limit of normal)§**	**Thyroid imaging**	**Other defects**	**Inheritance of genetic variant and phenotype in carriers**
ALGS_1 M/11 yrs	p.R937Q	<10	22.0	127	Hypoplasia (4.6 mL)	TGV, single heart ventricle, chronic liver failure and cholestasis, growth retardation	Paternal (no ALGS1 features in the family, father TSH 2.1 mU/L)
ALGS_2 M/25 yrs	p.T587I	<20	4.3	113	n.a	VSD and coarctation of the aorta, typical facial dysmorphism, cholestasis	Paternal (father with reminiscent facial dysmorphisms, TSH 5.4 mU/L)
ALGS_3 M/3 yrs	IVS_18 c.2860+1 delAG	<10	7.2	120	GIS (4.4 mL)	Persistent atrial septal defect, embryotoxon, butterfly vertebrae	Paternal (JAG1 variant co-segregated with ALGS1 phenotype in father and elder brother; TSH n.a.)
ALGS_4 F/10 yrs	p.N1026E*fs*X8	<10	5.6	140	GIS (6 mL)	Pulmonary artery stenosis, bile duct paucity, butterfly vertebrae	*de novo* (no ALGS1 features in the family)
ALGS_5 M/21 yrs	p.C917G	<20	9.7	136	n.a	Right ventricle hypoplasia, pulmonary artery atresia, posterior embryotoxon, mild cholestasis, absent gallbladder	Maternal (no ALGS1 features in the family)
ALGS_6 M/6 yrs	p.I1035X	n.a	15.3	105	Hypoplasia (2 mL)	Chronic cholestasis, typical facial dysmorphism, VSD	n.a.
CH1 F/3 wks	p.R937Q	>100	245	60	Apparent athyreosis^	Atrial septal defect	Paternal (TSH n.a.)
CH2 F/2 wks	p.R744Q	>100	812	13	Lingual ectopy	VSD and pulmonary artery atresia	Maternal (mother TSH 6.1 mU/L)

CH3 F/2 wks	p.R937Q	>100	>500	27	Apparent athyreosis^	none	n.a.
CH4 M/3 wks	p.R937Q	12	10.7	80	GIS	none	n.a. (mother TSH 4.8 mU/L)

Note: M, male; F, female; * at the diagnosis of hypothyroidism; yrs, years; wks, weeks; § normal values in ng/dL: 1.5-2.4 for infants, 0.9-2.0 for pre-pubertal children, 0.7-1.7 for adults; dbs, dry blood spot; TGV, transposition of the great arteries; VSD, ventricular septal defect; GIS, gland-in-situ; ^, gland not visible at scintiscan, but profound hypoplasia at ultrasound associated with detectable thyroid hormone in serum; n.a.; not available.

Figure

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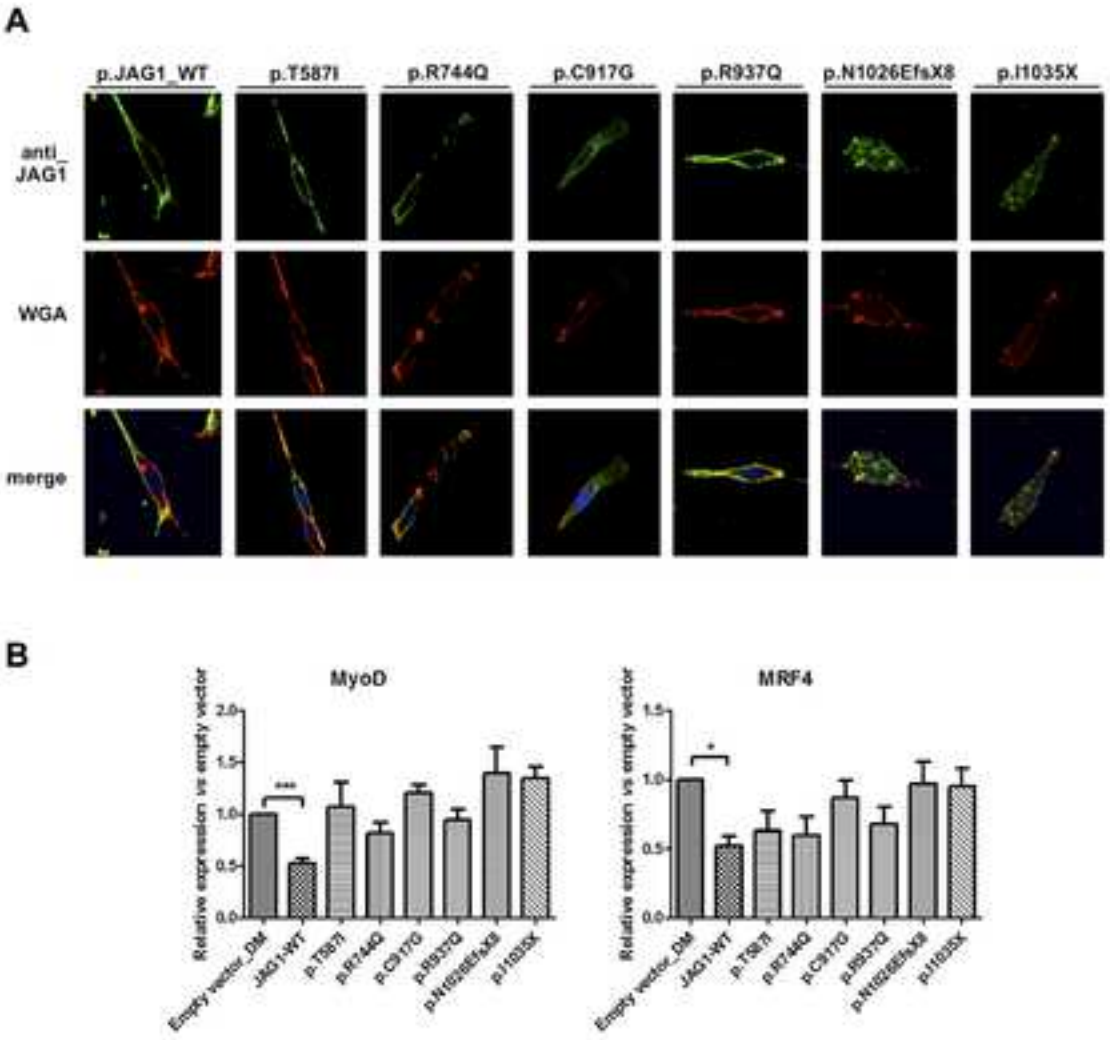

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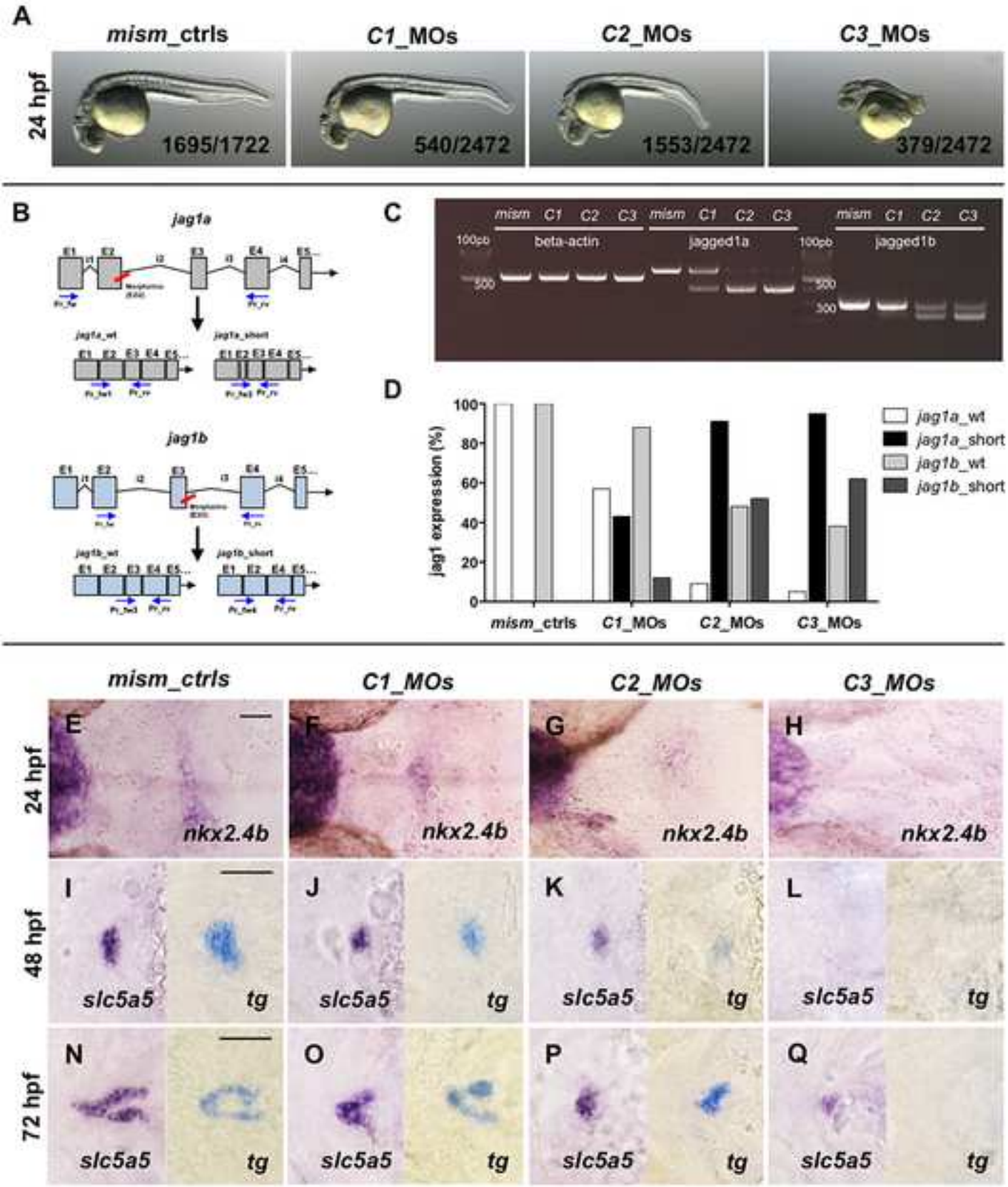

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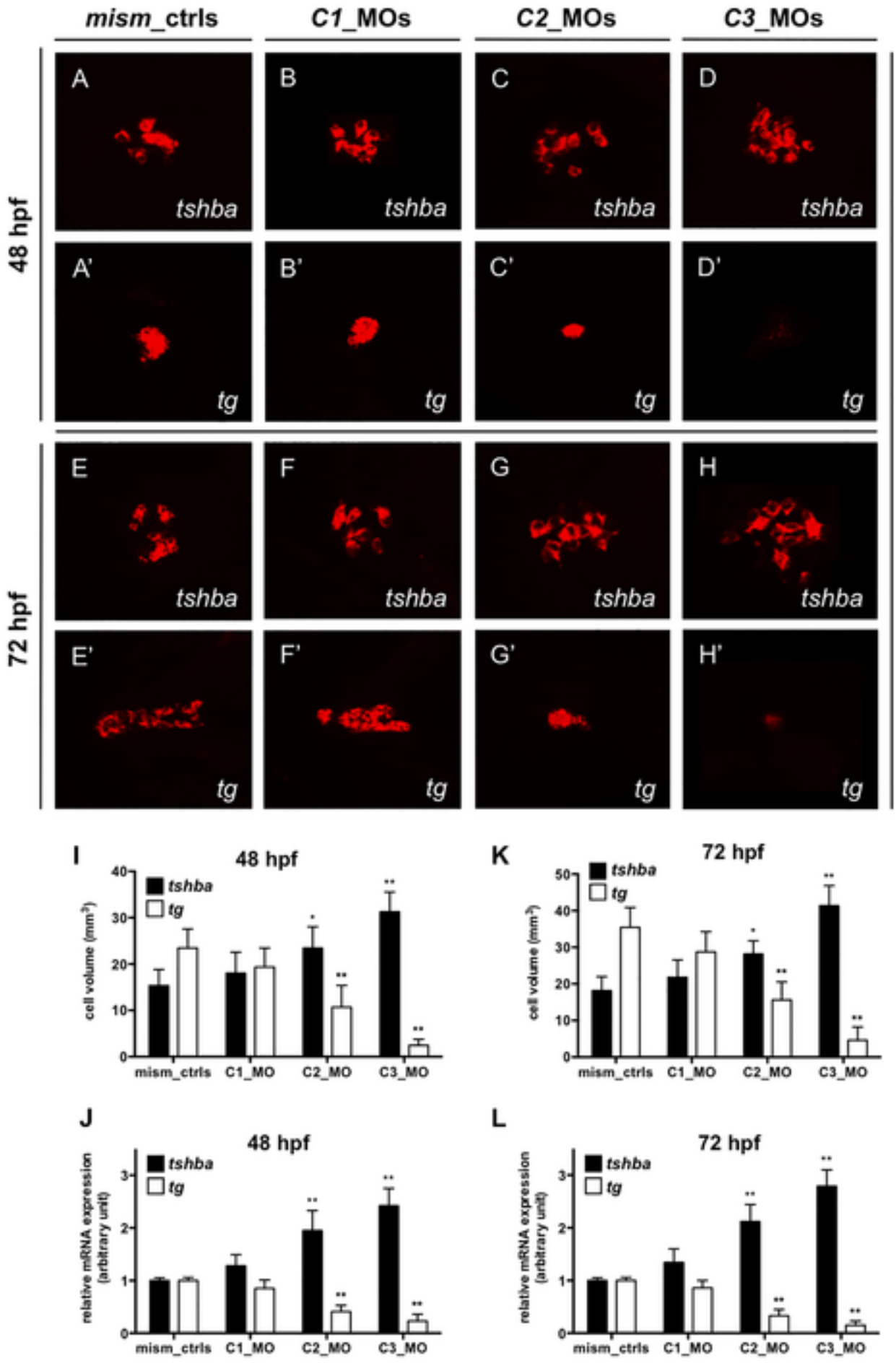

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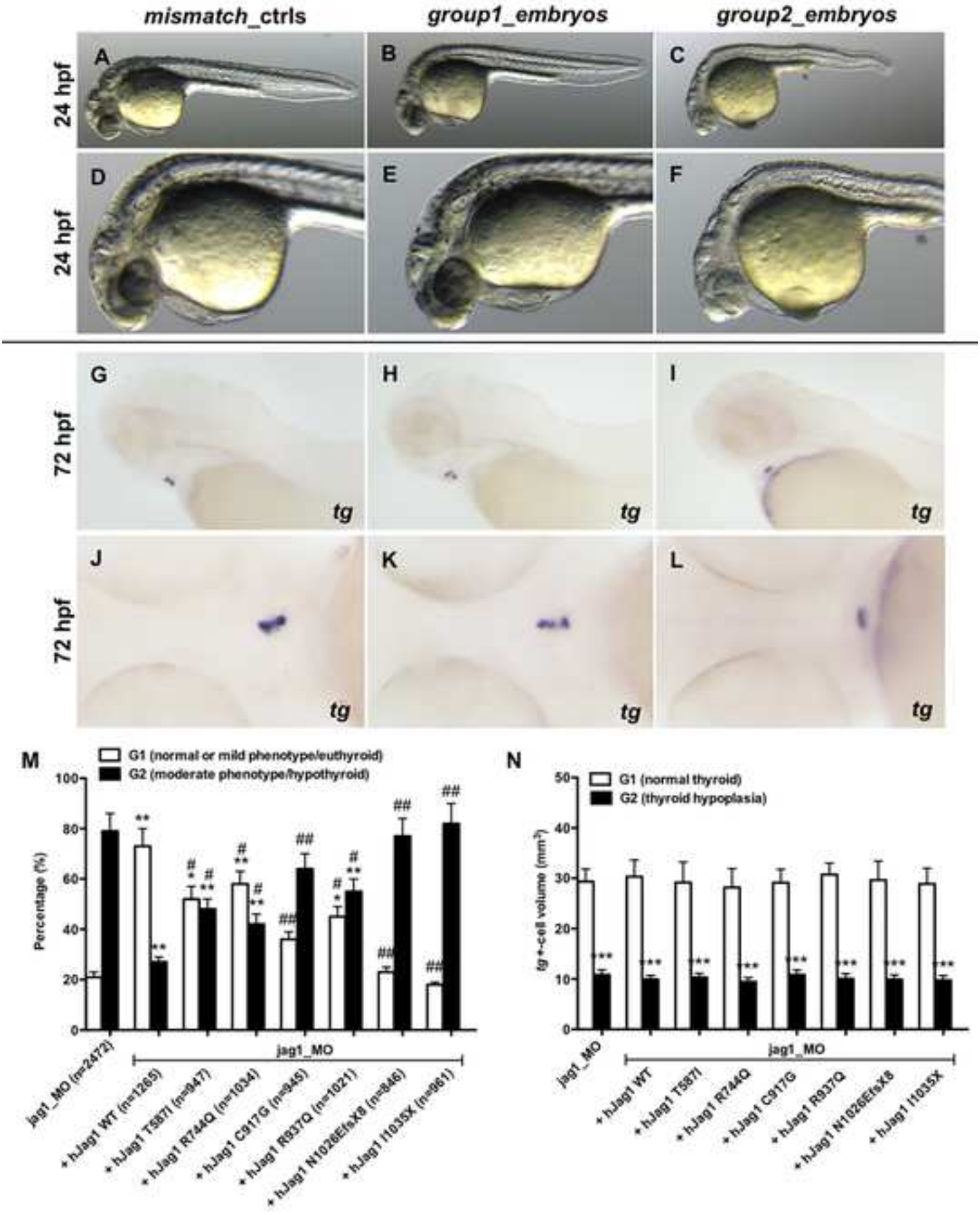

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