



HAL
open science

ALK2 mutation in a patient with Down syndrome and a congenital heart defect

Irene C Joziasse, Kelly A Smith, Sonja Chocron, Maarten van Dinther, Guryev Victor, Jasper van Der Smagt, Edwin Cuppen, Peter ten Dijke, Barbara Mulder, Cheryl L Maslen, et al.

► **To cite this version:**

Irene C Joziasse, Kelly A Smith, Sonja Chocron, Maarten van Dinther, Guryev Victor, et al.. ALK2 mutation in a patient with Down syndrome and a congenital heart defect. *European Journal of Human Genetics*, 2011, 10.1038/ejhg.2010.224 . hal-00609408

HAL Id: hal-00609408

<https://hal.science/hal-00609408>

Submitted on 19 Jul 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

ALK2 mutation in a patient with Down syndrome and a congenital heart defect

Running title: ALK2 mutation in Down syndrome and heart defect

Irene C. Joziasse, MD MSc PhD^{1,8}, Kelly A Smith, PhD^{2,3,8}, Sonja Chocron, BSc^{2,3},
Maarten van Dinther, BSc⁴, Victor Guryev, PhD², Jasper J van de Smagt, MD⁵,
Edwin Cuppen, PhD^{2,5}, Peter ten Dijke, PhD⁴, Barbara JM Mulder, MD PhD^{3,6},
Cheryl L Maslen, PhD⁷, Benjamin Reshey, Bsc⁷, Pieter A Doevendans, MD PhD¹,
Jeroen Bakkers, PhD^{2,3}

¹ Department of Cardiology, University Medical Center Utrecht, the Netherlands

² Hubrecht Institute and University Medical Center Utrecht, the Netherlands

³ Interuniversity Cardiology Institute of the Netherlands

⁴ Department of Molecular Cell Biology, Leiden University Medical Center and Center for Biomedical Genetics, Leiden, The Netherlands

⁵ Department of Medical Genetics, University Medical Center Utrecht, the Netherlands

⁶ Department of Cardiology Academic Medical Center, Amsterdam, The Netherlands

⁷ Division of Cardiovascular Medicine, Oregon Health & Science University, Portland, Oregon, USA

⁸ These authors contributed equally to this work

Correspondence address

Jeroen Bakkers, PhD

Associate Professor

Cardiac Development and Genetics

Hubrecht Institute

Email: j.bakkers@hubrecht.eu

Tel. +31.30.2121892

Fax. +31.30.2121801

ALK2 mutation in a patient with Down syndrome and a congenital heart defect

Abstract Down syndrome, resulting from an additional copy of chromosome 21 (trisomy 21), is frequently associated with congenital heart defects. Although increased dosage of chromosome 21 sequences are likely to be part of the etiology of cardiac defects, only a proportion of Down syndrome patients exhibit a congenital heart defect (birth prevalence 40-60%). Through a large candidate gene sequencing screen in patients with atrioventricular septal defects, substitutions were identified in bone morphogenetic protein (*BMP*) type I receptor *ALK2* and two other genes in a patient with Down syndrome and a primum type atrial septal defect. Structural modeling of the cytoplasmic domain of the *ALK2* receptor suggests that H286 is in close proximity to the nucleotide binding site of the kinase domain. We investigated whether this p.His286Asp substitution altered *ALK2* function by using both *in vitro* as well as *in vivo* assays. The p.His286Asp variant demonstrated impaired functional activity as measured by BMP-specific transcriptional response assays. Furthermore, mild dominant-interfering activity was observed *in vivo* compared with wild type *ALK2* as determined by RNA injection into zebrafish embryos. These data indicate that in the context of a Down syndrome background, *ALK2* mediated reduction of *BMP* signalling may contribute to congenital heart defects.

Keywords: Down Syndrome, Congenital Heart defects, gene mutation

Introduction

Down syndrome (DS) is the most frequent cause of mental retardation, most often caused by trisomy 21 (in approximately 95% of cases). DS occurs in about one in 700-1000 live births¹⁻³. The syndrome is characterized by well-defined and distinctive phenotypic features including characteristic facies, minor limb anomalies, hypotonia and growth retardation⁴. Whilst all individuals with DS manifest these phenotypes, there is significant phenotypic variability between DS individuals⁵. In conjunction with these phenotypic traits, DS individuals suffer a higher incidence of several conditions, including congenital heart defects (CHDs)⁵.

The (birth) prevalence of congenital heart defects in DS is about 40-60%, most frequently atrioventricular septal defects (AVSD) and ventricular septal defects (VSD)⁶⁻⁸. Efforts have been made to determine the critical chromosomal region for specific phenotypic features of DS by deletion mapping and characterization of patients with partial trisomy 21^{4, 9, 10}. One potential candidate gene for these trisomy 21-related CHD is collagen Type VI. However, the evidence for the involvement of collagen Type VI has, to date, been restricted to correlative expression pattern analysis^{11, 12}.

While this susceptibility and variability of the DS phenotype is widely accepted, the factors contributing to this variability have not been established. It is postulated that variations in gene dosage of chromosome 21, environmental factors and genetic modifications not linked to chromosome 21 account for this variability; and it is likely that contributions from each of these elements are involved. Genetic imbalance caused by the presence of an extra copy of chromosome 21 will seriously disrupt one or more developmental pathways. In addition the presence of non-chromosome 21

loci variation may predispose for a heart defect. To date, *CRELD1* (3p25.1) is the only non-chromosome 21 gene that has been reported to associate with DS-related CHDs¹³.

We have previously reported mutations in *ALK2*, a type I receptor for bone morphogenetic proteins (BMPs), in patients with endocardial cushion-associated CHDs. One of these variants identified has a dominant-interfering effect on BMP signaling and is associated with primum type atrial septal defect (ASD type I)¹⁴. Here we report an additional *ALK2* variant that was identified in a patient with trisomy 21 and a primum type atrial septal defect (ASD type I). In addition to the *ALK2* variant, two other variations were detected in another type I BMP receptor gene, *ALK3*, and in the epidermal growth factor receptor family, *ErbB3*. Using both *in vitro* and *in vivo* assays, we show that the *ALK2* variant has reduced BMP-inductive capacity and a mild dominant-interfering effect on BMP signaling. This effect on BMP signaling from the *ALK2* variation in conjunction with the additional variations identified in this patient is likely to contribute to the manifestation of a CHD in this individual.

Material and Methods

This study was prospectively reviewed and approved by the Local Research Ethics Committee of the University Medical Center Utrecht and the Academic Medical Center, Amsterdam, The Netherlands.

Genetic material and clinical evaluation

The patient described in this study was identified in a large-scale candidate gene sequencing screen as previously described¹⁴. In total 9 candidate genes were

sequenced in this patient (*ALK2*, *ALK3*, *EGFR*, *GATA4*, *HAS2*, *UGDH*, *ERBB2*, *ERBB3*, *CRELD1*). Patient material was acquired from the CONCOR (CONgenital CORvitia) database ¹⁵. DNA material was obtained from the patient and first degree relatives by buccal swabs. Family members were clinically evaluated and individuals testing positive for the *ALK2*, *ERBB3* and *ALK3* variant were examined by echocardiography. DNA material from patients with non-cardiac-related diseases (Fragile X [n=170], male infertility [n=90] and Rett Syndrome [n=90]) was used for controls. Controls were sourced from the Dutch population which comprises 80% of individuals of European ancestry (according to the Central Bureau of Statistics, The Netherlands, for 2008).

Detection of variants

PCR primers (sequence available upon request) for amplification were designed using an in-house developed management system (<http://limstill.niob.knaw.nl>), which was also used for the semi-automated identification and classification of mutations ¹⁶. Selected genes were analysed by dideoxy sequencing of PCR-amplified exonic fragments.

Cell lines and transfections.

Bovine aortic endothelial cells ¹⁷ were used for luciferase assays and cos7 cells were used for construct expression. All transfections were performed using Lipofectamine (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions.

Constructs, luciferase assay and kinase activity assays.

Expression constructs encoding full-length human ALK2 have been cloned into the pcDNA3 vector as previously described¹⁸ and mutant variants were generated by site-directed mutagenesis using a QuikChange kit (Stratagene, La Jolla, CA, USA). Inserts were fully sequenced and recloned into the original parental vector or the pCS2+ vector (for mRNA synthesis) to ensure against additional mutations. The luciferase assay was performed as previously described¹⁹. This included an injection with vector only or in combination with a short hairpin RNA (shRNA), the latter to knockdown endogenous bovine ALK2 but not vector-based human ALK2, as described previously¹⁴.

Zebrafish lines, mRNA synthesis and injections

Wild type fish were kept under standard conditions as previously described²⁰. pCS2+ ALK2 constructs were linearised with *NotI* enzyme and capped mRNA was prepared with the Message Machine kit (Ambion, Austin, TX, USA). mRNA was diluted to appropriate concentration in nuclease-free water and 1 nl²⁰ per embryo injected at the 1-2 cell stage. Embryos were imaged live and mounted in methylcellulose.

Statistical analysis

Results are expressed as mean \pm SEM. Statistical significance was determined by one-way analysis of variance, followed by a Bonferroni *t* test using the program SigmaStat version 2.03 (Jandel Scientific, San Rafael, CA).

Results

We recently performed a screen for the detection of cSNPs (coding single nucleotide polymorphisms) in patients with congenital heart defects arising from improper endocardial cushion formation¹⁴. During this screen we identified a patient with Down syndrome in possession of compound cSNPs in *ALK2* resulted in a His286Asp substitution (*ALK2* p.His286Asp) (Figure 1b); the *ALK3* cSNP caused a Glu414Lys substitution (*ALK3* p.Glu414Lys); whilst the cSNP in *ERBB3* resulted in a Thr1169Ile substitution (*ERBB3* p.Thr1169Ile) (Supplement figure 1). These cSNPs were analyzed with the prediction tools PolyPhen^{21, 22} and SIFT²³ (Table 1). Only *ALK2* p.His286Asp was predicted to damage protein function in both models and was not detected in 350 controls of the Dutch population. The coding regions of a total of 9 genes were sequenced in this individual, including *CRELD1*, and no other cSNPs were identified.

This patient with DS was diagnosed with a primum type atrial septal defect with a cleft mitral valve and the coronary sinus draining into the left atrium (Figure 1e). Sequencing of DNA from peripheral blood lymphocytes confirmed the 3 cSNPs in this individual (figure 1d). To determine heritability and segregation of the cSNPs, we screened all first degree relatives of the proband for the presence of each substitution (Figure 1d). The father of the proband was found to possess both the *ALK2* p.His286Asp and the *ALK3* p.Glu414Lys variants whereas the mother was a carrier of the *ERBB3* p.Thr1169Ile variant. Of the proband's two siblings, only the sister carried the *ALK3* p.Glu414Lys and the *ERBB3* p.Thr1169Ile variants whereas the brother was negative for the three cSNPs. None of these relatives were found to have a CHD by cardiac echocardiography.

We utilised our previous experience in functionally characterising ALK2 variants to investigate the ALK2 p.His286Asp variant. Structural modelling of the cytoplasmic domain of the ALK2 receptor demonstrated that H286 resides within the ATP binding pocket of the kinase domain, indicating that a missense substitution at this residue may impair receptor activity (Figure 1a,c). To test this hypothesis, ALK2 p.His286Asp was examined for its capacity to induce BMP-specific transcriptional reporter activity¹⁹. In this reporter assay the ALK2 p.His286Asp variant showed significantly lower luciferase activity with and without BMP6 induction compared with wtALK2 ($p < 0.05$) (Figure 2a). Next, endogenous ALK2 expression was knocked down by an RNAi construct as previously described¹⁴. Transfection with the ALK2 p.His286Asp variant demonstrated significantly lower levels of luciferase activity in the uninduced state ($p < 0.05$) but a difference from wtALK2 was not observed upon stimulation with BMP6 (Figure 2b). These results indicate that the ALK2 p.His286Asp receptor is capable of propagating a signal when stimulated with a BMP ligand, but this activity is impaired when the ALK2 p.His286Asp variant is in the presence of the wtALK2 form of the receptor.

To investigate the effect of the ALK2 p.His286Asp variant on receptor function *in vivo*, synthetic wtALK2 and ALK2 p.His286Asp mRNAs was injected into zebrafish embryos at the single cell stage. Injection of the ALK2 p.His286Asp variant resulted in mild dorsalisation of the zebrafish (C1 in 17% versus 3% of injection with wtALK2; $p < 0.05$)²⁴, which demonstrates a mild dominant-interfering effect of the ALK2 p.His286Asp variant on BMP signalling in the gastrulating embryo (Figure 3a). As a further measure of the reduction of ALK2 signalling for the ALK2

p.His286Asp variant, rescue experiments were performed in the zebrafish *ALK2/8* mutant, *lost-a-fin (laf)*, which is identifiable for its C1 dorsalisation phenotype (Figure 3c). While both wtALK2 and variant ALK2 mRNA were able to rescue the C1 dorsalization phenotype in *laf/alk2* embryos, the ALK2 p.His286Asp did so less effectively than wt (51% versus 65%, respectively; $p < 0.05$; Figure 3a).

Discussion

We recently reported a dominant-negative *ALK2* allele in a patient with a congenital heart defect¹⁴. In the current study we report a patient with Down syndrome and a primum type atrial septal defect who harbours three cSNPs in genes associated with endocardial cushion development. The cSNPs result in missense substitutions in the type I BMP receptors genes, *ALK2* and *ALK3* resulting in the genetic variants p.His286Asp and p.Glu414Lys, respectively, and in the epidermal growth factor receptor family gene, *ERBB3*, resulting in the p.Thr1169Ile variant

We show that, whilst subtle, there is a statistically significant difference in BMP signalling activity in *ALK2* p.His286Asp compared with the wtALK2 allele by both *in vitro* and *in vivo* analysis. Additionally, a genetic variant in *ALK3* was identified. How these compound substitutions in two distinct type I BMP receptors affect overall BMP signalling was not tested here but could affect BMP signalling to a greater extent when present in a compound manner, since *ALK2* and *ALK3* can form a heterodimeric complex²⁵. Furthermore, it is difficult to predict what adverse effects may arise when occurring in concert with the non-conservative *ERBB3* p.Thr1169Ile substitution. What can be determined from the family data is that none of these

variants are sufficient to cause a CHD when present in isolation in the absence of trisomy 21.

The three variants were detected in the parents of the probands demonstrating that these variants were not acquired *de novo*. The father of the proband carries the *ALK2* p.His286Asp and *ALK3* p.Glu414Lys variants and the sister of the proband harbours the *ALK3* p.Glu414Lys and the *ERBB3* p.Thr1169Ile variants. Neither of these individuals presented with a CHD, demonstrating that the possession of either of these two combinations of cSNPs is either not sufficient to cause the heart defect or the variants are not completely penetrant.

Our data are consistent with the notion that *ALK2* is a susceptibility gene for primum type atrial septal defects, in combination with variants at other loci, or in combination with trisomy 21. This case is also suggestive that additional predisposing genetic factors exist for heart defects in DS patients thus underlining the polygenic nature of these defects. These genetic factors can be identified by large scale candidate gene screens.

Acknowledgements

We kindly acknowledge the cooperation of the index patient and his family members.

Conflict of interest

All authors state that they have no conflicting interests to declare.

Reference List

1. Bell R, Rankin J, Donaldson LJ: Down's syndrome: occurrence and outcome in the north of England, 1985-99. *Paediatr Perinat Epidemiol* 2003; **17**: 33-9.
2. Mikkelsen M, Fischer G, Stene J, Stene E, Petersen E: Incidence study of Down's syndrome in Copenhagen, 1960-1971; with chromosome investigation. *Ann Hum Genet* 1976; **40**: 177-82.
3. Owens JR, Harris F, Walker S, McAllister E, West L: The incidence of Down's syndrome over a 19-year period with special reference to maternal age. *J Med Genet* 1983; **20**: 90-3.
4. Korenberg JR, Chen XN, Schipper R, *et al*: Down syndrome phenotypes: the consequences of chromosomal imbalance. *Proc Natl Acad Sci USA* 1994; **91**: 4997-5001.
5. Wiseman FK, Alford KA, Tybulewicz VL, Fisher EM: Down syndrome--recent progress and future prospects. *Hum Mol Genet* 2009; **18**: R75-R83.
6. Loffredo CA, Hirata J, Wilson PD, Ferencz C, Lurie IW: Atrioventricular septal defects: possible etiologic differences between complete and partial defects. *Teratology* 2001; **63**: 87-93.
7. Paladini D, Tartaglione A, Agangi A, *et al*: The association between congenital heart disease and Down syndrome in prenatal life. *Ultrasound Obstet Gynecol* 2000; **15**: 104-8.

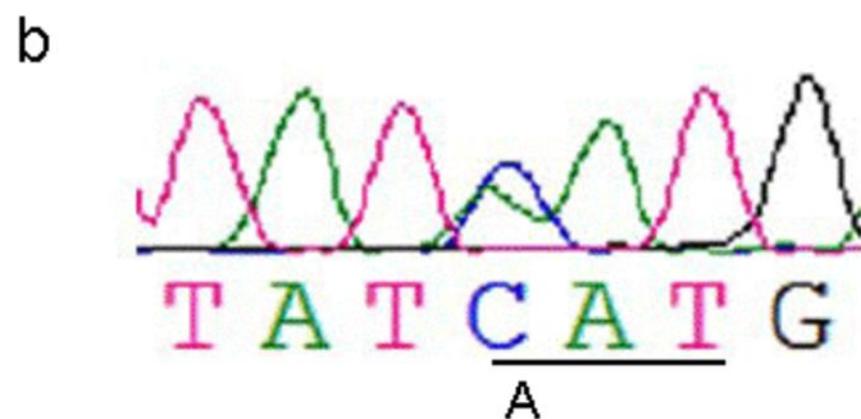
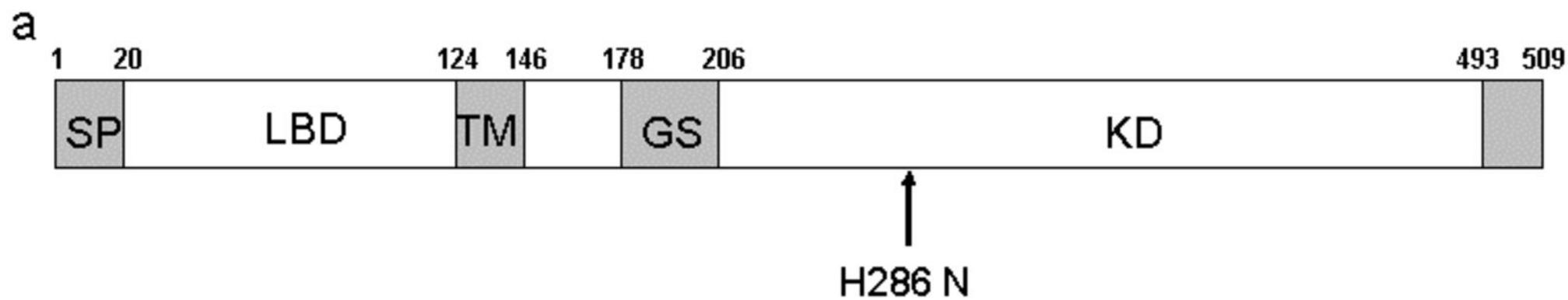
8. Vis JC, Duffels MG, Winter MM, Weijerman ME, Cobben JM, Huisman SA, Mulder BJ. Down syndrome: a cardiovascular perspective. **J Intellect Disabil Res** 2009; **53**: 419-25.
9. Korenberg JR, Kawashima H, Pulst SM, *et al*: Molecular definition of a region of chromosome 21 that causes features of the Down syndrome phenotype. *Am J Hum Genet* 1990; **47**: 236-46.
10. Kerstann KF, Feingold E, Freeman SB, *et al*: Linkage disequilibrium mapping in trisomic populations: analytical approaches and an application to congenital heart defects in Down syndrome. *Genet Epidemiol* 2004; **27**: 240-51.
11. Klewer SE, Krob SL, Kolker SJ, Kitten GT: Expression of type VI collagen in the developing mouse heart. *Dev Dyn* 1998; **211**: 248-55.
12. Gittenberger-de Groot AC, Bartram U, Oosthoek PW, *et al*: Collagen type VI expression during cardiac development and in human fetuses with trisomy 21. *Anat Rec A Discov Mol Cell Evol Biol* 2003; **275**: 1109-16.
13. Maslen CL, Babcock D, Robinson SW, *et al*: CRELD1 mutations contribute to the occurrence of cardiac atrioventricular septal defects in Down syndrome. *Am J Med Genet A* 2006; **140**: 2501-5.
14. Smith KA, Joziassse IC, Chocron S, *et al*: Identification of a dominant-negative ALK2 allele in a family with congenital heart defects. *Circulation* 2009; **119**: 3062-9.
15. van der Velde ET, Vriend JW, Mannens MM, *et al*: CONCOR, an initiative towards a national registry and DNA-bank of patients with congenital heart

- disease in the Netherlands: rationale, design, and first results. *Eur J Epidemiol* 2005; **20**: 549-57.
16. Prandini P, Deutsch S, Lyle R, *et al*: Natural gene-expression variation in Down syndrome modulates the outcome of gene-dosage imbalance. *Am J Hum Genet* 2007; **81**: 252-63.
 17. Hammerschmidt M, Mullins MC: Dorsoventral patterning in the zebrafish: bone morphogenetic proteins and beyond. *Results Probl Cell Differ* 2002; **40**: 72-95.
 18. ten Dijke P, Yamashita H, Ichijo H, *et al*: Characterization of type I receptors for transforming growth factor-beta and activin. *Science* 1994; **264**: 101-4.
 19. Korchynskiy O, ten Dijke P: Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. *J Biol Chem* 2002; **277**: 4883-91.
 20. Westerfield M: *The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio)*. 4th ed. Eugene: University of Oregon Press; 2000.
 21. Sunyaev S, Ramensky V, Bork P: Towards a structural basis of human non-synonymous single nucleotide polymorphisms. *Trends Genet* 2000; **16**: 198-200.
 22. Sunyaev S, Ramensky V, Koch I, Lathe W 3rd, Kondrashov AS, Bork P: Prediction of deleterious human alleles. *Hum Mol Genet* 2001; **10**: 591-7.
 23. Ng PC, Henikoff S: SIFT: Predicting amino acid changes that affect protein function. *Nucleic Acids Res* 2003; **31**: 3812-4.

24. Mullins MC, Hammerschmidt M, Kane DA, *et al*: Genes establishing dorsoventral pattern formation in the zebrafish embryo: the ventral specifying genes. *Development* 1996; **123**: 81-93.
25. Little SC, Mullins MC: Bone morphogenetic protein heterodimers assemble heteromeric type I receptor complexes to pattern the dorsoventral axis. *Nat Cell Biol* 2009; **11**: 637-43.

Table 1. Predictions of ALK3 and ErbB3 variant using PolyPhen and SIFT.

	PolyPhen	SIFT
ALK3 p.Glu415Lys	Benign variant	Affect protein function
ErbB3 p.Thr1169Ile	Benign variant	Alter protein function (low confidence)



Human	L	I	T	H	E	M	G	S	L
Mouse	L	I	T	H	E	M	G	S	L
Chick	L	I	T	H	E	M	G	S	L
Zebrafish	L	I	T	H	E	M	G	S	L

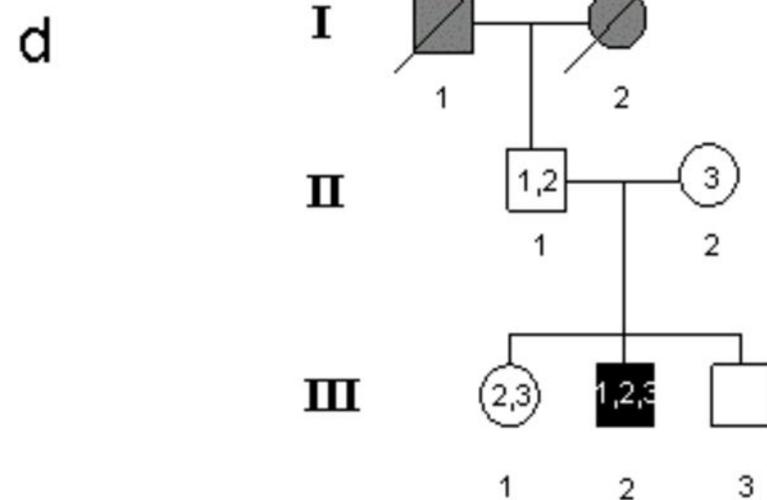
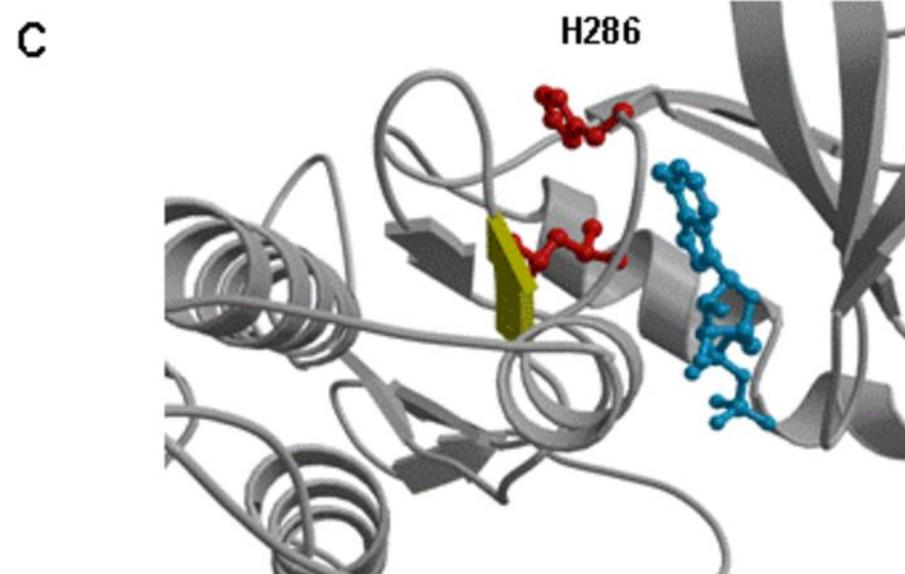


Figure 2

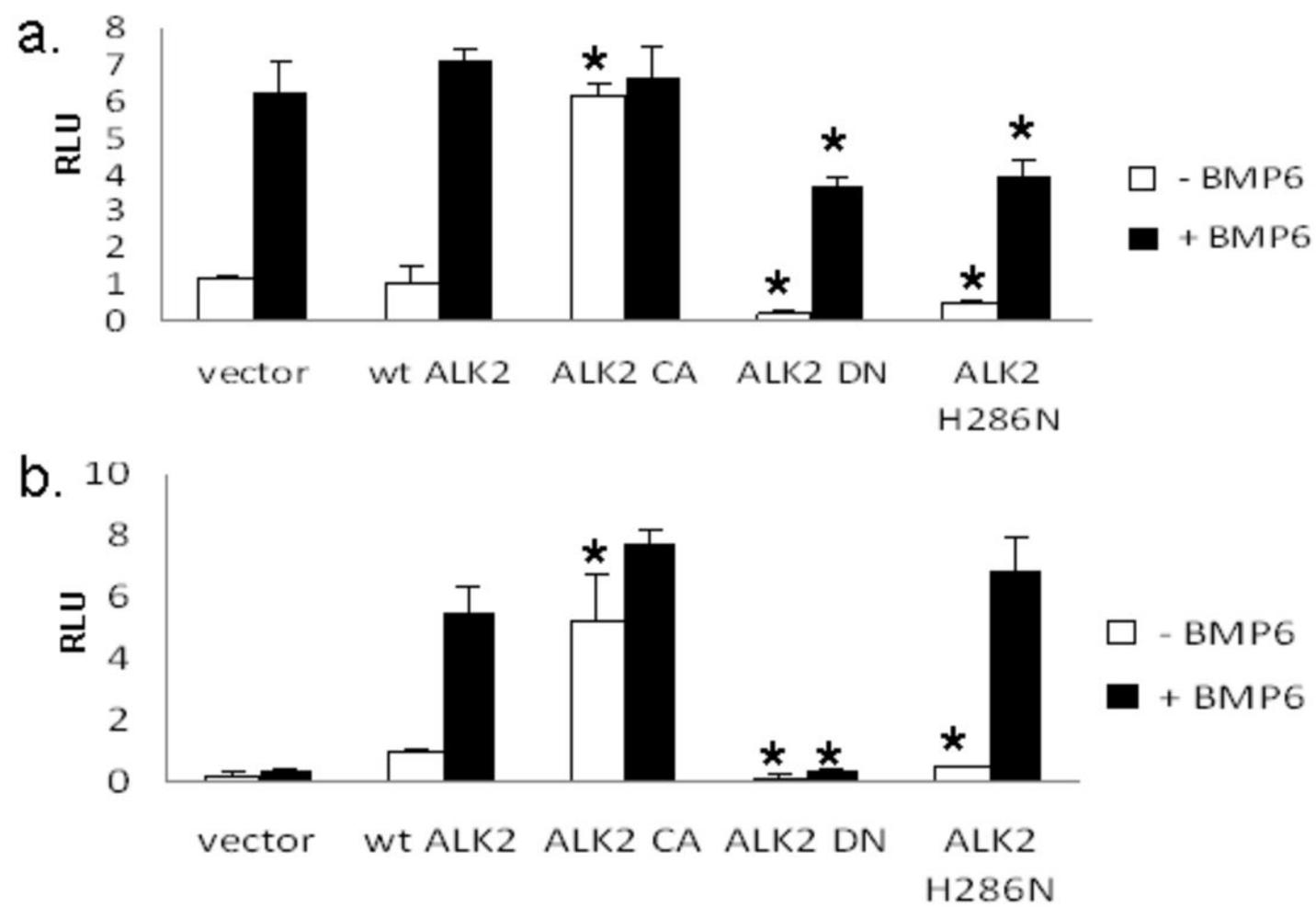
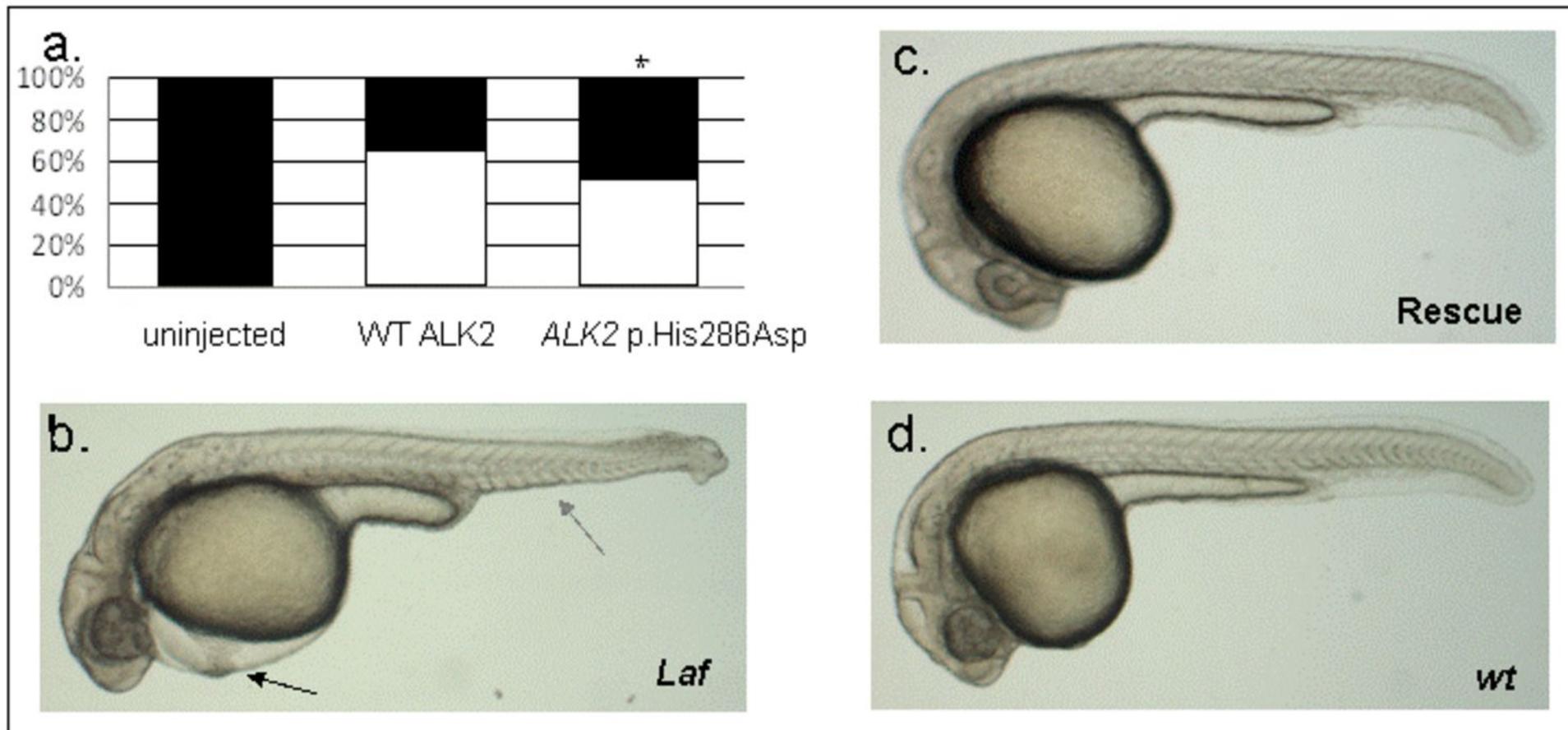
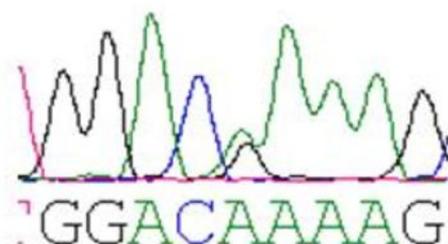


Figure 3



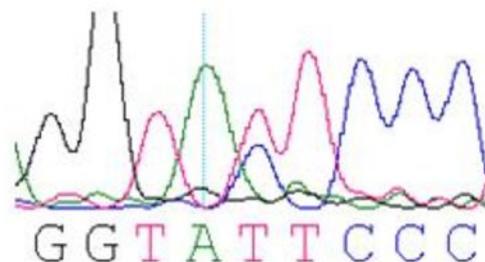
ALK3 E415K



G

Human	PEVLD	E	SLNKN
Mouse	PELVD	E	SLNKN
Chick	PEVLD	E	SLNKN
Zebrafish	PELVD	E	SLNKN

ErbB3 T1169I



C

Human	THLKG	T	PSSRE
Mouse	THLKG	T	PSSRE
Chick	CAGR-	T	PGPTG
Zebrafish	SPEKE	T	LSSRS