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Zebrafish as a model to study the role of *PAX5/ETV6* fusion gene in Acute Lymphoblastic Leukemia and the function of the single wild type genes in normal hematopoiesis

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Part I

1. Abstract

B cell precursors Acute Lymphoblastic Leukemia (BCP-ALL) is a tumor characterized by the malignant expansion of B cell progenitors and it is the most common childhood cancer. About 30% of BCP-ALL cases are characterized by mutations, deletions or translocations of the *PAX5* gene. Among *PAX5* translocations, the most recurrent is t(9;12) which determines the generation of *PAX5/ETV6* fusion gene. The PAX5 transcription factor is essential for B cell lineage commitment and differentiation. PAX5 fulfills its function mainly through the activation of B lineage-specific genes and the repression of inappropriate ones. ETV6 transcription factor is specifically required for adult hematopoiesis in mice. Moreover, ETV6 accelerates erythroid differentiation of cell lines and it stimulates hemoglobin synthesis in adult mice. PAX5/ETV6 is able to down-regulate the expression of some PAX5-activated-genes, as well as to up-regulate some PAX5-repressed-genes. Accordingly, PAX5/ETV6 caused the alteration of B cell differentiation and conferred survival advantages to mouse B cell precursors *in vitro*. The acquisition of survival advantages by PAX5/ETV6 expressing cells is due to the activation of the Lck-STAT5 pathway leading to the upregulation of STAT5 effectors *Ccnd2* and *cMyc*. When we started this project very little was known about the conservation of *pax5* and *etv6* roles in zebrafish hematopoiesis. We thus analyzed their expression pattern and we found that these genes are expressed in several hematopoietic tissues. Indeed, *pax5* is expressed in pancreas, which has been proposed to be a transient embryonic site of B cell lymphopoiesis in zebrafish. *pax5* knockdown caused the alteration of the expression of several B cell markers. Otherwise, *etv6* is expressed in tissues related to both primitive and definitive hematopoiesis. *etv6* loss of function determined the reduction of primitive mature erythrocytes and we found that this alteration is due to the defective differentiation of primitive erythrocytes. Moreover, we tried to shed light into the mechanism through which *etv6* regulates primitive erythrocytes maturation. Interestingly, we found that *etv6* is a potential modulator of Notch signaling in primitive erythrocytes. Furthermore, *etv6* is involved, directly or indirectly, in the repression of *klf1, klf3, klf6a* and *klf17* genes, which are essential for primitive erythrocytes maturation. Overall, our findings suggest that the roles of *pax5* and *etv6* in hematopoiesis could be, at least in part, conserved in zebrafish. Interestingly, *etv6* knockdown caused the alteration of the expression of some B cell markers which are impaired also by *pax5* loss of function, thus, we investigated if the contemporary *pax5* and *etv6* knock-down could cooperate to alter the expression of these genes. However, the coinjection of *pax5* and *etv6* morpholinos did not cause significant alteration of *igh*μ and *cd79a*, thus excluding a possible cooperation between *pax5* and *etv6* in the regulation of their expression. Finally, we

transiently expressed *PAX5/ETV6* in zebrafish embryos and, despite it did not caused the deregulation of B cell markers expression, this aberrant gene is able to activate the same pathway which is altered *in vitro*, inducing the phosphorylation of Lck and Stat5 proteins as well as the upregulation of Stat5 effectors, suggesting that zebrafish could be a suitable model to study BCP-ALL related to *PAX5/ETV6* fusion gene.

2. State of the Art

Acute Lymphoblastic Leukemia (ALL) is a tumor characterized by the malignant expansion of lymphoid progenitor cells. The 85% of ALL cases are caused by the proliferation of B cell precursors and this subtype of leukemia is indeed called B cell precursors ALL (BCP-ALL) (Guzman and Jordan, 2004). BCP-ALL is the most common childhood cancer and the main cause of death related to tumor in children and young adults (Mullighan, 2012). About 30% of BCP-ALL cases are characterized by mutations, deletions or translocations of *PAX5* gene. Among several translocations involving different partner genes, the most recurrent is t(9;12) that causes the fusion of *PAX5* with *ETV6* gene (Mullighan *et al.,* 2007; An *et al.,* 2008; Kawamata *et al.,*2008). Despite several genetic lesions involving both *PAX5* and *ETV6* have been identified, we are fare from the complete understanding of their role in the pathogenesis of leukemia. Moreover, the information currently available derive mainly from *in vitro* models. However, these models are obviously unable to fully represent the complexity of animal organisms and is thus necessary to reproduce human pathologies *in vivo* for two purposes: gain new insights in the molecular mechanisms underlying diseases to discover novel therapeutic targets and perform drug screenings to find new pharmacological treatments.

Zebrafish has emerged as an excellent model organism because it allows to link genetic and molecular studies to morphological whole-organism analysis and it offers several advantages compared to other vertebrate model systems. The external fertilization and development, together with the optical clarity of the embryos allow *in vivo* and *ex vivo* easy visualization of embryonic development and manipulation of zebrafish embryos. Moreover, the small size of the zebrafish embryo ensures a sufficient supply of oxygen by passive diffusion to survive and develop for several days in condition of absence of blood circulation. Zebrafish resulted to be particularly suitable for the study of hematopoiesis and hematopoietic disorders affecting humans, because the developmental processes and genetic programs of hematopoiesis, as well as the main blood cell types, are highly conserved, despite some differences do exist (Lieschke and Currie, 2007; Paik and Zon, 2010; Jing and Zon, 2011). Zebrafish models of human leukemia caused by *ETV6/RUNX1* and *ETV6/JAK2* gene fusions have already been generated and they were found to be useful tools to gain insights into the leukemic process *in vivo* and for the identification of new potential therapeutic targets (Sabaawy *et al.,* 2006; Onnebo *et al.,* 2012).

The main aims of this project are to test if the roles of *pax5* and *etv6* in hematopoiesis are conserved in zebrafish and to verify if zebrafish could be a useful model to reproduce *PAX5/ETV6* related leukemia.

2.1. Hematopoiesis

Hematopoiesis is the process by which blood cells are formed and occurs during embryonic development and throughout adulthood to produce and renew the blood cellular elements. the developmental processes and genetic programs of hematopoiesis are highly conserved between fish and mammals, although some differences do exist. All vertebrates, included zebrafish, are characterized by two main wave of hematopoiesis. During the first one, called primitive wave, transient precursors give rise only to erythrocytes and macrophages during early embryonic development; during the second one, called definitive wave, multipotent hematopoietic stem cells (HSCs) gives rise to all blood cell lineages (Fig.1; Galloway and Zon, 2003).

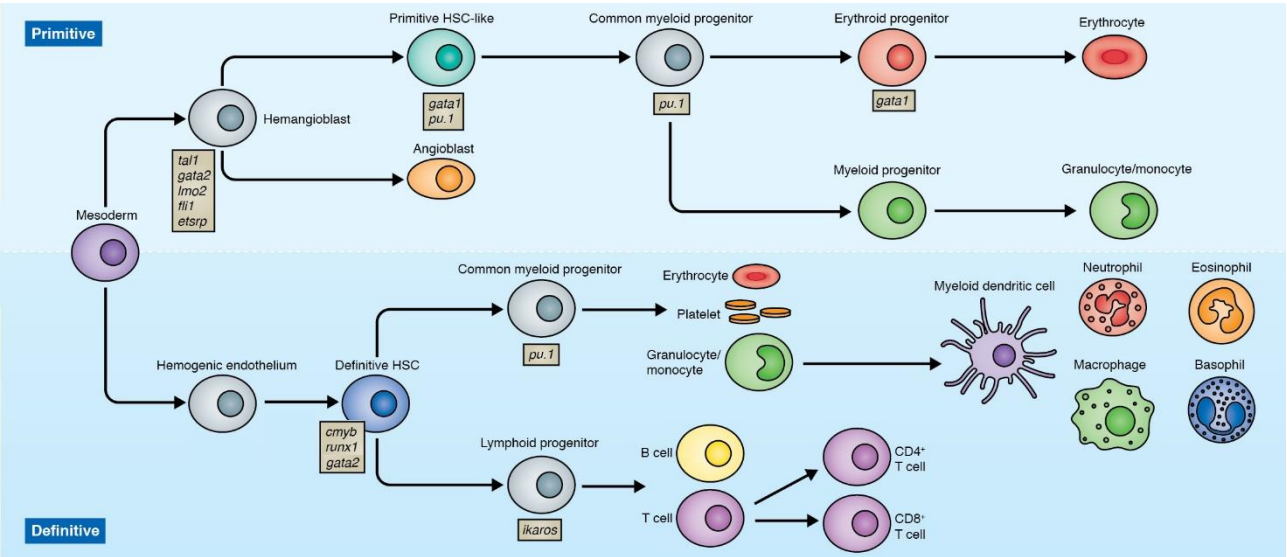

Figure 1: Primitive and definitive waves in zebrafish. Some of the most important genes are represented. HSC: hematopoietic stem cell (modified from Jagannathan-Bogdan and Zon, 2013).

The primary purpose of the primitive wave is to produce red blood cells that can facilitate tissue oxygenation as the embryo undergoes rapid growth (Orkin and Zon, 2008). In mammals and birds, primitive hematopoiesis occurs in the blood islands located in the extra-embryonic yolk sac, whereas in zebrafish primitive blood cells originate in intra-embryonic structures (Detrich *et al.,* 1995; Palis *et al.,* 2001). Primitive myelopoiesis takes place in the anterior lateral mesoderm (ALM), located in the rostral portion of the embryo, and mainly produces macrophages (Fig. 2; Herbomel *et al.,* 1999). Primitive erythroid precursors originate from two bilateral stripes in the posterior lateral plate mesoderm (PLM) around 5 somite stage (Fig. 2; Detrich *et al.,* 1995). Later, around 18 somite stage, these precursors migrate towards the midline to give rise to the equivalent of the mammalian blood islands, the intermediate cell mass (ICM). Within the ICM, erythroid progenitors differentiate in proerytrhroblasts that enter the circulation at 24-26 hpf (hours post fertilization; Fig. 2; Detrich *et al.,* 1995). These cells continue to mature in circulation and are the only circulating red cell population through 4dpf (days post fertilization), but can persist until at least 7 dpf (Weinstein *et al.,* 1996; Chen and Zon, 2009). Erythrocytes differentiation consists in cytological changes in cell size, in nuclear shape and in cytoplasm staining. The most immature cells are big round-shaped, have a large and open nucleus and a more basophilic cytoplasm (Ransom et al., 1996). Mature erythrocytes are rugby-ball shaped and smaller than precursors, the nucleus is more condensed and the cytoplasm is acidophilic (Ransom et al., 1996). All zebrafish mature erythrocytes, both primitive and definitive, are nucleated, while mammalian erythrocytes retain their nucleus in the primitive wave but not in the adult (Chen and Zon, 2009). These cytological changes are accompanied by 'maturational' globin switching as erythrocytes differentiate. The first switch take place between 24 hpf and 48 hpf, and it is characterized by the sharp decrease of *hemoglobin beta embryonic 3* (*hbbe3*) gene and the concomitant increase of *hbbe1* gene. The second switch that establishes the adult *globin* expression begins around 22 dpf leading to the increasing expression of the nearly exclusively adult *globins αa1* and *βa1* by 32 dpf (Ganis *et al.,* 2012).

Primitive hematopoieis is largely regulated by *gata1*, the master regulator gene of erythrocyte development, and *pu*.1, crucial for myeloid fate acquisition (Scott *et al.,* 1994; Cantor *et al.,* 2002). These two transcription factors exhibit a cross-inhibitory relationship to regulate erythrocyte and myeloid fates. Other important transcriptional regulators of erythropoiesis are the members of the Kruppel-like transcription factor family, that are known to activate erythroid-specific genes (Palis, 2014). In mammals KLF1 primarily regulates the adult *β GLOBIN* gene but also the expression of multiple erythroid-specific genes, whereas in zebrafish it is known to be involved in primitive erythropoiesis regulating *α globin* gene (Donze *et al.,* 1995; Hodge *et al.,* 2006; Pilon *et al.,* 2006;

Fu *et al.*, 2009). Moreover, in zebrafish *klf3* and *klf6a* are essential for erythroid cell differentiation and maturation (Xue *et al.*, 2015). Also *klf17*, previously reported as *klf4*, is essential for primitive erythrocytes maturation through the activation of genes involved in heme synthesis and embryonic globin genes. Specifically, *klf17* binds to the embryonic *β-like globin* gene promoters and *klf17* loss of function causes the downregulation of *hbbe3* gene (Gardiner *et al.*, 2007; Xue *et al.*, 2015).

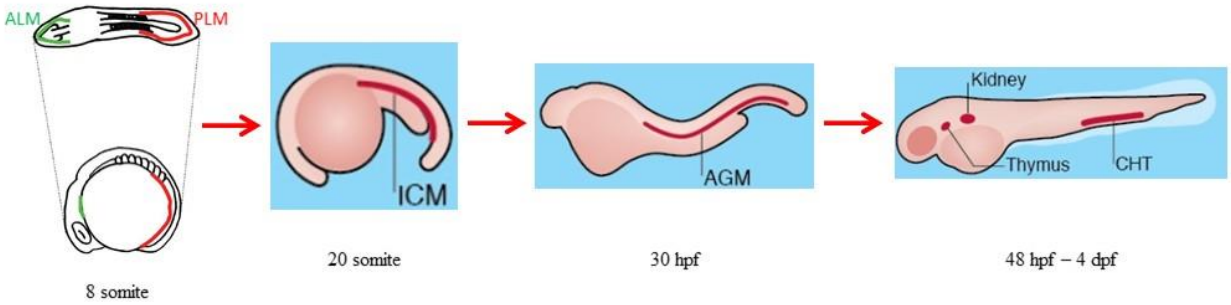

Figure 2: Sites of hematopoiesis during zebrafish development. Sites of primitive hematopoiesis: anterior lateral mesoderm (ALM) and posterior lateral mesoderm (PLM); intermediate cell mass (ICM), Sites of definitive hematopoiesis: aorta-gonad-mesonephros region (AGM); caudal hemtopoietic tissue (CHT); kidney; thymus. The corresponding developmental stages are shown. Some of the most important genes are represented. HSC: hematopoietic stem cell (modified from Paik and Zon, 2010; Jagannathan-Bogdan and Zon, 2013)

Definitive hematopoiesis provides long-term multipotent HSCs with self-renewal property, which are able to differentiate in erythroid, myeloid and lymphoid lineages throughout the life of the organism. In mammals HSCs originate from the aorta-gonad-mesonephros region (AGM) and they migrate to the fetal liver and then to the bone marrow, which is the location for HSCs in adults (Fig. 2; Cumano and Godin, 2007). In zebrafish, the ventral wall of the dorsal aorta has been identified as the analogous of this AGM region (Murayama *et al.*, 2006; Kissa *et al.*, 2008). Starting from about 30 hpf, HSCs emerge from the ventral wall of the dorsal aorta, they enter circulation through the axial vein and migrate to the region of the caudal hematopoietic tissue (CHT) in the posterior region of the tail (Fig. 2; Bertrand *et al.*, 2010; Kissa and Herbomel, 2010). Around 3 dpf, HSCs leave the CHT to colonize the organs that will be the sites of adult hematopoiesis. HSCs colonize first the thymus, the site of T cell lymphopoiesis, and one day later the kidney marrow, the site of hematopoiesis of all the other blood cell lineages, which is the analogous of the bone marrow in mammals (Fig. 2; Murayama *et al.*, 2006; Jin *et al.*, 2007; Bertrand *et al.*, 2008; Kissa *et al.*, 2008).

2.1.1. B cell development

The pluripotent HSCs with its self-renewal potential regenerates all blood cell types throughout life by differentiating to progenitor cells with gradually restricted developmental potential generating a large number of specialized cells. One of the earliest event in hematopoiesis is the differentiation of HSCs in the multipotent progenitor (MPP) which can undergoes commitment to either the lymphoid or erythro-myeloid lineages, resulting in the formation of the early lymphoid progenitors (ELP) or common myeloid progenitors (CMP Fig. 4). ELPs start to express the *recombination activating gene 1* (*rag1*) and *rag2* and initiate rearrangements at the *immunoglobulin heavy chain* (*IgH*) locus. The ELP can further differentiate into thymic precursors of the T-cell lineage or into bone-marrow common lymphoid progenitors (CLP; Fig. 4). The CLPs can generate B cells, T cells, dendritic cells and natural killer cells. In mammals, the entry of CLPs in the B cell differentiation pathway and the transition to the pro-B stage is characterized by the expression of the B cell marker B220 (Fig.4). Subsequently, CD19 is expressed and the *IgH* diversity (D_H)-to-joining (J_H) gene segment rearrangement is completed, identifying pre-BI cells. The *IgH* locus then continues to rearrange its variable (V_H) region gene segments until productive V_H–DJ_H alleles are generated in large pre-BII cells. These cells expose the product of the rearranged *IgH* gene at the cell surface, where it assembles with the co-receptors molecules Igα, encoded by the *Cd79a* gene, and Igβ, encoded by the *Cd79b* gene, to form the pre-B-cell receptor (pre-BCR; Fig. 3, 4).

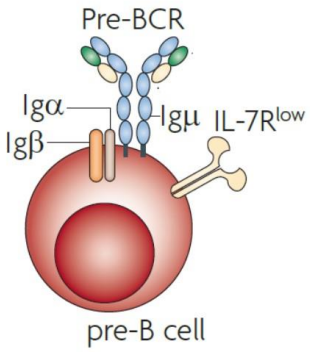

Figure 3: Schematic representation of a pre-B cell exposing the pre-BCR. (Modified from Herzog *et al.*,2009).

The expression of the pre-BCR is a crucial check-point in early B-cell development, and its signaling stimulates a proliferative clonal expansion of large pre-BII cells, which is followed by the rearrangements of immunoglobulin light-chain genes. Once an immature B cell expresses IgM on its surface, it emigrates from the bone marrow to peripheral lymphoid organs, such as the spleen, where

they further differentiate through several transitional stages. After the appropriate selection, transitional B cell can differentiate into mature B cells. About 10% of mature splenic B cells are marginal zone B cells. They are strategically positioned at the blood-lymphoid tissue interface, where they can initiate a fast and vigorous antibody response to blood pathogens. Transitional, follicular and even memory B cells can give rise to marginal-zone B cells, responsible for antibody response to blood pathogens, and/or be recruited into this compartment. In mice, most mature B cells are follicular B cells. These cells are mainly responsible for generating humoral immune responses to protein antigens. With the help of T cells, they form germinal centres. Germinal-centre B cells proliferate rapidly, undergo somatic hypermutation of their immunoglobulin variable gene segments and undergo isotype-switch recombination of immunoglobulin genes. Subsequently, germinal centres slowly vanish, and memory B cells and effector plasma cells are generated (Busslinger, 2004; Matthias and Rolink, 2005).

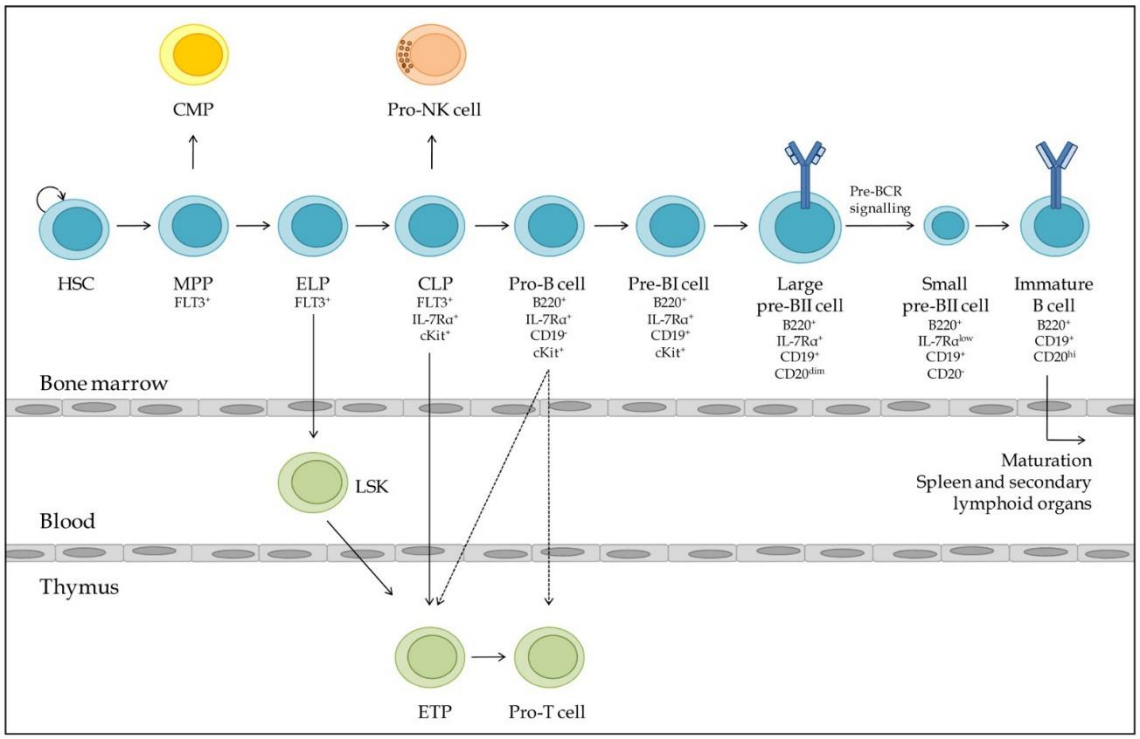

Figure 4: Schematic representation of B cell development. The main markers of the different maturation stages are indicated. Hematopoietic stem cell (HSC); multipotential progenitor (MPP); early lymphoid progenitor (ELP); common lymphoid progenitor (CLP); common myeloid progenitor (CMP); early T progenitor (ETP) (Fazio *et al.*,2011).

During early B cell development, three transcription factors have been found to be essential for the differentiation of CLPs into specified pro-B cells: *E2A*, *EBF1* and *PAX5*. Absence of any of these factors leads to an early block in B cell development at the pro-B cell or pre-B cell stage. These three factors seem to work in collaboration, and together, they form a master control switch for engaging B cell differentiation (Matthias and Rolink, 2005). E2A and EBF1 are considered primary B cell fate determinants and coordinately activate the expression of B cell specific genes, however, they are not sufficient to commit B cell progenitor to B cell lineage (Fuxa and Skok, 2007).

Very little is known about B cell lymphopoiesis during zebrafish development, in fact, Only Danilova and Steiner (2002) suggested that the pancreas could be a transient site of B cell lymphopoiesis in zebrafish. This hypothesis is based on the expression of *rag1* at 4 dpf and *igh*μ from 10 dpf in the pancreas. Subsequently, B cell lymphopoiesis moves to the head kidney at about 2 weeks post fertilization (wpf), as suggested by *rag1* expression in this organ. Accordingly, *igh*μ expression has been observed in the kidney at 19 dpf (Danilova and Steiner, 2002; Zapata *et al.*, 2006).

2.2. Acute Lymphoblastic Leukemia

Acute Lymphoblastic Leukemia (ALL) is a biologically heterogeneous disorder characterized by the malignant proliferation of lymphoblasts, the lymphoid progenitor cells, which are not able to properly differentiate (Guzman and Jordan, 2004). The disease is characterized by the clonal proliferation and accumulation of malignant blast cells in the bone marrow and peripheral blood (Harrison, 2001). An estimated 6000 new cases of ALL are diagnosed every year in the USA, patients are both adults and children but ALL incidence peaks between 2 and 5 years of age (Inaba *et al.,* 2013). ALL is mainly caused by the acquisition of mutations in somatic cells and only less than 5% of the cases are associated with inherited genetic syndromes, such as Down's syndrome (Pui *et al.,* 2008; Inaba *et al.,* 2013). The most of childhood cases of ALL show extended chromosomal alterations such as: hyperdiploidy; hypodiploidy; recurring translocations leading to aberrant gene fusions such as *BCR-ABL1* and *ETV6-RUNX1*; rearrangements of *MLL* with a wide range of partner genes and rearrangements of *MYC* (Inaba *et al.,* 2013). Several of this genetic lesions disrupt genes involved in hematopoiesis (*RUNX1, ETV6*), activate oncogenes (*MYC*) and constitutively activate tyrosine kinases (*ABL1*). However, many of these alterations alone are not sufficient to induce leukemia in experimental models, suggesting that other genetic lesions cooperate to oncogenic transformation; for example, pathologies related to *ETV6-RUNX1* and *BCR-ABL1* have between six and eight

additional genetic alterations (Fig. 5; Greaves *et al*., 2003; Mullighan *et al*., 2007; Pui *et al.,* 2008; Inaba *et al.,* 2013). Several of these cooperating mutated genes encode tumor suppressors, cell cycle and lymphoid development regulators and are responsible for the establishment of the specific leukemic clone. The most frequently mutated genes, affecting about 40% of BCP-ALL cases, are transcription factors involved in B cell development such as *PAX5, E2A, EBF1* and *IKAROS* that can be altered by various mechanisms including deletions, amplifications, sequence mutations and translocations (Fig. 5; Mullighan *et al*., 2007; Pui *et al.,* 2008; Inaba *et al.,* 2013). In some case these transcription factors are functionally normal but their expression is deregulated leading to abnormal proliferation and block of differentiation of lymphoid progenitors. In other case the chromosomal translocations generate chimeric transcription factors which can alter the expression of normal targets of the endogenous proteins and cause the recognition of genes different than those recognized by wild type factors (Mullighan *et al*., 2007; O'Neil and Look, 2007; Pui *et al*., 2008; Inaba *et al*., 2013).

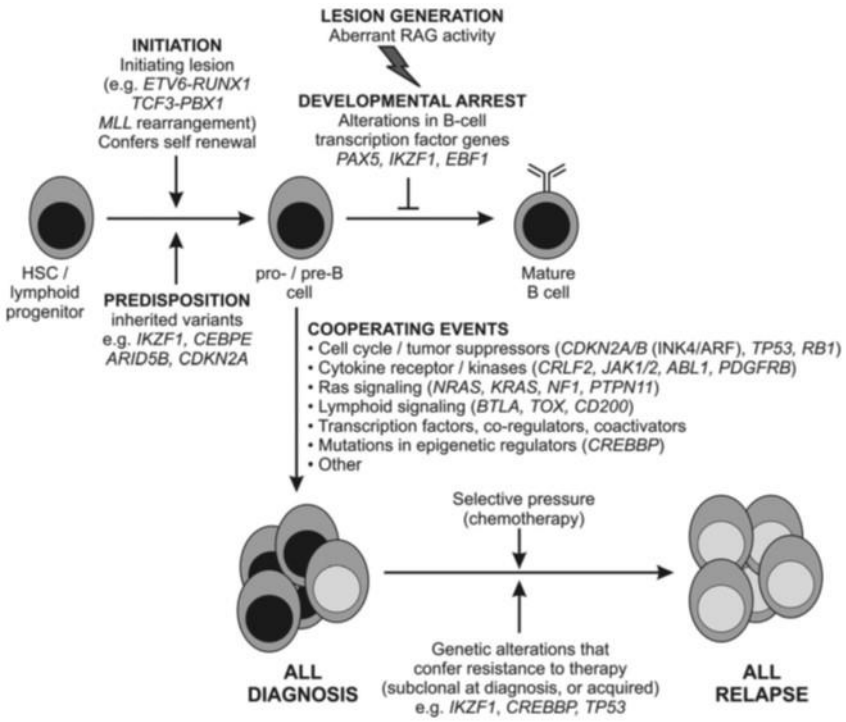

Figure 5: Proposed model for the role of genetic alterations in BCP-ALL. (Mullighan, 2012).

Overall, these findings suggested a multistep model of leukemia pathogenesis in which sequential mutational events are accumulated starting from the initial alteration, usually acquired *in utero*, through the largely covert evolution to overt disease, that can occur even several years later after the acquisition of the initial lesion (Fig. 5; Greaves *et al*., 2003; Mullighan, 2012; Inaba *et al.,* 2013).

Now it is commonly thoughts that acute lymphoblastic leukaemia, like cancer in general, probably arises from interactions between exogenous or endogenous exposures, genetic (inherited) susceptibility, and chance. The challenge is to identify the relevant exposures and inherited genetic variants and decipher their roles in leukemia pathogenesis (Fig. 5; Greaves *et al*., 2003; Mullighan, 2012; Inaba *et al.,* 2013).

About 15% of ALL cases are a result of the proliferation of T cell precursors, whereas the remaining 85% of the cases are caused by the expansion of B cell precursors (Guzman and Jordan, 2004). The *PAX5* gene is the most frequent target of somatic mutations in BCP-ALL cases. Specifically, about 30% of BCP-ALL cases are characterized by mutations, deletions or translocations of *PAX5*; the most recurrent among *PAX5* translocations is t(9;12), that causes the fusion of *PAX5* with *ETV6* gene (Cazzaniga *et al*., 2001; Mullighan *et al*., 2007).

2.3. *PAX5*

2.3.1. *PAX5* gene and encoded protein structure

PAX5 (*Paired box domain* 5) belongs to the Paired Box family of transcription factors which comprises 9 genes in mammals, 15 in zebrafish and 4 in the basal chordates such as the amphioxus (Stapleton *et al.,* 1993; Paixao-Cortes *et al*., 2013). This diversity in the number of *PAX* genes across chordates is due to three event of whole genome duplication, two occurred during early vertebrate evolution and one teleost-specific, and other additional partial duplication events (Amores *et al.,* 1998; Meyer and Van de Peer, 2005; Van de Peer *et al.,* 2009; Paixao-Cortes *et al*., 2013). Human *PAX5* is located on the chromosome 9 and has 10 exons which can give rise to more than 10 splicing variants in both normal and cancer cells; it has been suggested that alternative splicing could be an important mechanism of regulation of PAX5 activity (Zwollo *et al.,* 1997; Borson *et al.,* 2002; Arseneau *et al.,* 2009). Zebrafish *pax5* is mapped on chromosome 1 and has 11 exons; two alternative splicing variants have been identified so far, the first is the full length coding sequence whereas the second one lacks the exon 2 and has a partial Paired Box DNA binding domain (Pfeffer *et al.,* 1998; Kwak *et al.,* 2006).

PAX proteins can be subdivided into four subfamilies based on the similarity of the Paired Box domain sequence and the presence of additional functional domains; According to these criteria, PAX5 is grouped into the PAX2/5/8 subfamily (Pfeffer *et al.,* 1998; Paixao-Cortes *et al*., 2015). Indeed, the defining feature of PAX5 is the Paired Box DNA binding domain (PD), located at the N-

terminal portion of the protein, which consists of an N-terminal and a C-terminal subdomain (Fig. 6; Czerny *et al.*, 1993). Each subdomain is composed of three helices organized in a helix-turn-helix motif and independently binds to a distinct half-site of the PAX5 recognition sequence in adjacent major grooves of the DNA helix (Garvie *et al.*, 2001). Each half-site independently contributes to the overall affinity of a given binding site and this characteristic of the PD domain is responsible for its degenerate consensus sequence (Fig. 6; Czerny *et al.*, 1993; Cobaleda *et al.*, 2007). The transcriptional activity of PAX5 is regulated by the interaction of several partners with central and C-terminal domains of the protein. Transactivation activity of PAX5 is mediated by the partial Homeodomain (HD) and the Transactivation domain (TAD) (Fig. 6; Cobaleda *et al.*, 2007). The partial HD of PAX5 interact with the TATA-binding protein of the basal transcription machinery and the Retinoblastoma protein, while the TAD modulates transcription probably through the interaction with histone acetyltranferases such as the Creb binding protein or SAGA complex (Fig. 6; Dorlfler and Busslinger, 1996; Eberhard and Busslinger, 1999; Emelyanov *et al.*, 2002; Barlev *et al.*, 2003). However, PAX5 can be converted into a transcriptional repressor by the Octapeptide (OP) domain and the Inhibitory domain (ID) (Fig. 6; Cobaleda *et al.*, 2007). In fact, the OP domain interact with corepressors of the Groucho protein family, which are component of a histone deacetylase complex (HDAC), whereas the ID negatively regulates the adjacent TAD (Fig. 6; Dorlfler and Busslinger, 1996; Eberhard *et al.*, 2000). All the functional domain of human PAX5 are highly conserved in zebrafish (Pfeffer *et al.*, 1998).

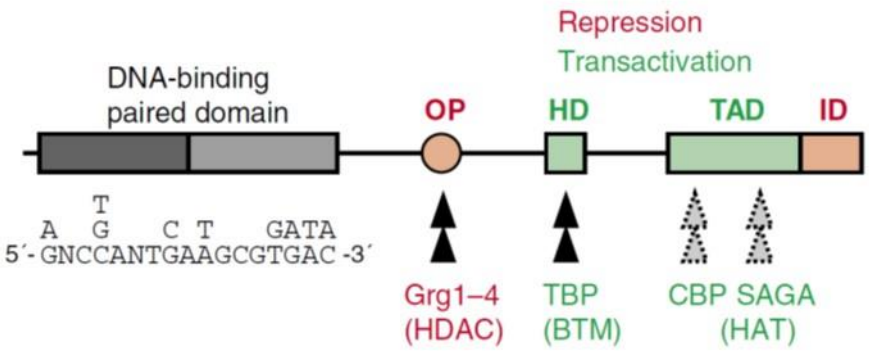

Figure 6: Functional domains and interacting protein of Pax5. The consensus recognition sequence of Pax5 is shown. Dashed gray arrows indicate a likely but not yet proven interaction between the Pax5 transactivation domain and the CREB-binding protein (CBP) or SAGA complex. Grg1–4 refers to the four full-length members of the mammalian Groucho protein family. Basal transcription machinery (BTM); histone acetyltransferase (HAT); partial homeodomain (HD); histone deacetylase (HDAC); inhibitory domain (ID); octapeptide domain (OP); transactivation domain (TAD); TATA-binding protein (TBP) (Cobaleda *et al.*, 2007).

2.3.2. *PAX5* role in embryonic development

During mouse embryo development *Pax5* is transiently expressed in the mesencephalon at embryonic day (E) 11,5 and in the neural tube at E 12,5; Starting from E 13,5 *Pax5* expression shifts to fetal liver, one of the sites of hematopoiesis in murine fetus, where it correlates with the onset of B lymphopoiesis (Adams *et al.,* 1992). In adult mouse *Pax5* is expressed in the testis and in several lymphoid tissues such as blood, spleen and lymph nodes (Adams *et al.,* 1992). Accordingly, *Pax5* is expressed in several cell lines representing the various B cell differentiation stages from pro-B to the mature B cell stages, however, *Pax5* is not expressed in plasma cells, the terminal stage of B cell differentiation, and all other hematopoietic cell lines analyzed (Barberis *et al.,* 1990; Adams *et al.,* 1992). Pax5 is the only Pax family member expressed in hematopoietic system and its important role in development has been shown by the generation of a *Pax5* knockout mouse model. The *Pax5* mutant mice usually die within three weeks after birth because of severe developmental defects of the central nervous system; moreover, B cell development in the bone marrow is arrested at an early precursor stage, the pro-B (or pre-BI) stage (Urbanek *et al.,* 1994; Nutt *et al.,* 1997).

During zebrafish embryonic development *pax5* is expressed in the brain, as in mouse and in *Xenopus laevis* (Adams *et al*., 1992; Short *et al*., 2012), specifically in the midbrain-hindbrain boundary from the 5 somite stage onwards (Fig. 7; Pfeffer *et al.,* 1998). Later, *pax5* expression is observed in the anterior part of the otic vesicle from the 17 somite stage to 72 hpf (Fig. 7; Pfeffer *et al.,* 1998). Interestingly, *pax5* is expressed in the otic vesicle also in *Xenopus laevis*, whereas in mouse it is not, thus suggesting that *Pax5* expression in the otic vesicle has not been conserved in Amniotes during evolution (Adams *et al*., 1992; Pfeffer *et al.,* 1998; Short *et al*., 2012).

To understand the role of *pax5* during zebrafish embryonic development Kwak and colleagues performed knock-down experiments by the injection of different morpholinos (Kwak *et al.,* 2006). Morpholinos are antisense oligonucleotides designed to specifically bind the target mRNA and block protein translation, when using translation blocking morpholinos, or alter pre-mRNA splicing when using splicing blocking morpholinos (Nasevicius and Ekker, 2000; Eisen and Smith, 2008). Embryos injected with *pax5* morpholinos (morphants) did not present any obvious phenotypical and morphological defects (Kwak *et al.,* 2006). However, the analysis of vestibular-dependent functions of balance, motor coordination and swim bladder inflation displayed that about 20% of morphant embryos showed severely impaired vestibular function, while acoustic function appeared normal, thus indicating that *pax5* is implicated in the development of the utricular macula and it is essential for vestibular function (Kwak *et al.,* 2006).

Figure 7: *pax5* expression during somitogenesis in zebrafish embryos. *pax5* is expressed in the midbrain-hindbrain boundary (mhb) and in the hindbrain (h) at 5 somites. At 20 somites it is expressed in the mhb and in the otic vescicle (Modified from Pfeffer *et al.,* 1998).

2.3.3. *PAX5* role B cell development

Now it is known that Pax5 is essential for several aspects of B cell development including: the commitment of B cell progenitors, the maintaining of cell identity throughout B lymphopoiesis until mature B cell stage and the immunoglobulin rearrangement (Cobaleda *et al.,* 2007).

B cell lineage commitment from the CLP depends on the basic-helix-loop-helix protein E2A, the Early B cell Factor 1 (EBF1), and Pax5 that function in a complex transcriptional network. The essential role of *Pax5* in commitment of B cell precursors has been elucidated with a pro-B (or pre-B) cell line obtained from *Pax5*$^{-/-}$ mice. These cells are able to differentiate into functional macrophages, granulocytes, dendritic cells, osteoclasts and natural killer cells both *in vitro*, when cultured with lineage-appropriate cytokines, and *in vivo*, upon transplantation into recipient mice; however, *Pax5*$^{-/-}$ cells can differentiate into mature B cells only after restoration of *Pax5* expression consequently to retroviral transfection (Fig. 8; Nutt *et al.,* 1999; Schaniel *et al.,* 2002).

Pax5 is fundamental not only for the commitment of B cell precursors but it is also essential for the maintaining of lineage identity throughout the differentiation process until mature B cell stage. In fact, conditional *Pax5* deletion in mature B cell causes the de-differentiation back to early uncommitted pro-B progenitor *in vivo* (Fig. 8; Cobaleda *et al.,* 2007b).

To ensure the commitment and the maintaining of B cell identity Pax5 fulfils a dual role activating the expression of B cell specific genes and repressing the inappropriate ones. Pax5 activate the expression of several genes which are essential for B cells such as: *Ebf1*, *Cd19*, *Cd79a* and *Blnk* (Fig. 9; Cobaleda *et al.,* 2007). *Ebf1* encodes another transcription factor that is necessary for B cell precursor commitment and it is in turn able to activate *Pax5* expression (Roessler *et al.,* 2007); CD19 protein is a co-receptor of the BCR (Kozmik *et al.,* 1992); *Cd79a* encodes the BCR component Igα

(Nutt *et al.*, 1998); Blnk (B cell linker) is a cytosolic protein involved in the signal transduction of the BCR (Schebesta *et al.*, 2002). There are several genes involved in the development of other blood cell lineages which must be repressed to allow the differentiation of B cell lineage (Fig. 9). Some of those repressed by Pax5 are: *Csf1r*, *Notch1* and *Lck*. *Csf1r* encodes the macrophage colony stimulating factor receptor (Nutt *et al.*, 1999); *Notch1* encodes a transmembrane receptor involved in T cell precursors specification (Souabni *et al.*, 2002). *Lck* encodes a tyrosine kinase involved in T cell development (Cobaleda *et al.*, 2007).

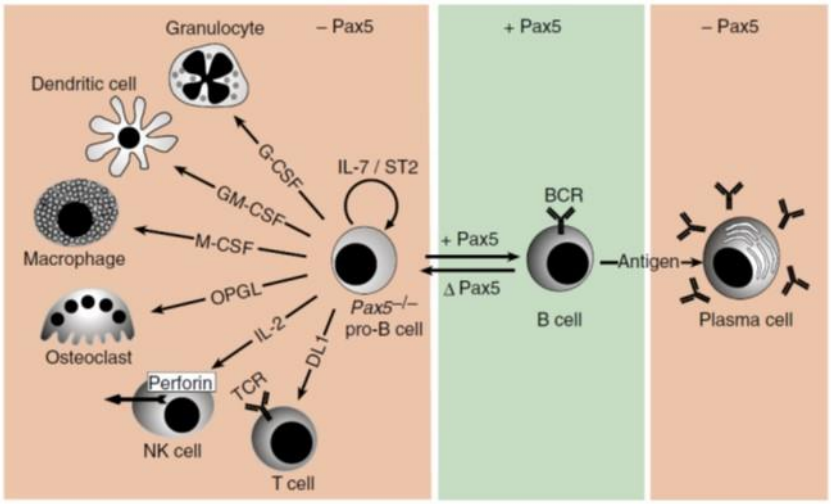

Figure 8: B cell lineage commitment by Pax5. The uncommitted Pax5$^{-/-}$ pro-B cells are able to differentiate into several hematopoietic cell types either *in vitro* in the presence of the indicated cytokines or *in vivo* after transplantation into recipient mice. Conditional Pax5 deletion (ΔPax5) results in retrodifferentiation of B lymphocytes to an uncommitted progenitor cell stage (Cobaleda *et al.*, 2007).

The progress of B cell maturation, especially the transition from pro-B to pre-B stage, depends on the proper signaling of the pre-BCR. One crucial step for the formation of a functional pre-BCR is the productive recombination of the *immunoglobulin heavy chain* locus (*Igh*), that results in the cell surface expression of the Igμ protein as a part of the pre-BCR (Busslinger *et al.*, 2004). The *Igh* locus is composed of three discontinuous segments: variable (V), diversity (D) and joining (J); V(D)J recombination occurs sequentially with D_H-J_H rearrangements preceding V_H-DJ_H recombination and it is controlled at several steps by various transcription factors (Busslinger *et al.*, 2004). Pax5 is important not only for the activation of *Cd79a* and *Blnk* expression, as previously mentioned, but also for the functional recombination of the *Igh* locus. In *Pax5*$^{-/-}$ pro-B cells D_H-J_H rearrangement proceeds

correctly, however V_H-DJ_H recombination are reduced 100-fold compared to wild-type pro-B cells. Hence, Pax5 is involved in regulating the spatial organization of the *Igh* locus and it exerts this function inducing the contraction of the locus thus promoting V_H-DJ_H recombination by facilitating connection formation between distal V_H and proximal D_H gene segments (Nutt *et al.*, 1997; Hesslein *et al.*,2003)

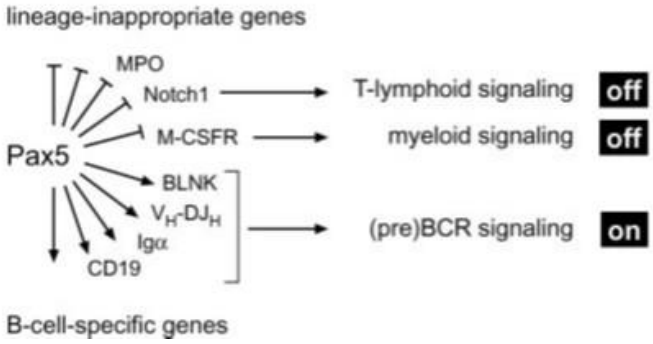

Figure 9: The role of Pax5 in B cell development. Pax5 represses lineage inappropriate genes and simultaneously activate B cell specific genes (Busslinger, 2004).

2.4. *PAX5* in hematological malignancies

PAX5 was initially associated with hematological malignancies as target of genetic alterations in lymphomas. The translocation t(9;14)(p13;q32) brings one allele of *PAX5* under the control of strong enhancers or promoters from the *IgH* locus and it has been associated with diffuse large B cell lymphomas (DLBCL), which are germinal-center B cell-derived tumors, and non-Hodgkin's lymphoma (Busslinger *et al.*, 1996; Lida *et al.*,1996; Morrison *et al.*, 1998). These observations suggest that if the PAX5-dependent gene expression program is altered, due to increased *PAX5* transcription in B cells or failed *PAX5* repression at the onset of plasma cell differentiation, the consequence could be tumor formation (Cobaleda *et al.*, 2007).

Despite lymphomas, *PAX5* aberrancies have been found in ALL. *PAX5* is located on the short arm of chromosome 9 which is subject to alterations, more commonly wide spread deletions, in about 10% of childhood ALL (Harrison, 2001). Notably, *PAX5* gene is the most frequent target of somatic alterations in BCP-ALL, being affected in about 30% of pediatric and 34% of adult cases (Mullighan *et al.*, 2007; Familiades *et al.*, 2009). *PAX5* alterations include: deletions, point mutations, amplifications and translocations.

The most frequent *PAX5* alteration is deletion, that occur in about 25% of cases, and is divided into three types: wide-range deletion involving *PAX5* and even the whole short arm of chromosome 9; focal deletions involving a subset of *PAX5* exons extending to its 3' region, thus leading to a prematurely truncated protein; focal deletions involving only a subset of internal PAX5 exons determining different PAX5 isoforms lacking functional domains (Mullighan *et al.,* 2007). *PAX5* deletions seem to be secondary events because they are frequently associated with other alterations such as *ETV6/RUNX1*, *BCR*/*ABL1* or *TCF3*/*PBX1*, both in adult and pediatric cases (Mullighan *et al.*, 2007; Familiades *et al.*, 2009).

PAX5 point mutations characterize about 7% of both adult and childhood cases (Mullighan *et al.*, 2007; Familiades *et al.,* 2009). Modelling studies using the *PAX5* crystal structure suggested that each of the nine point mutations founded in the paired box DNA binding domain should impair DNA binding activity. Also the transactivation domain is affected by point mutations, that consist of frameshift, splice site, or missense mutations (Mullighan *et al.,* 2007). Despite mutations associated to ALL are usually acquired in somatic cells, it has been reported the existence of a recurrent germline *PAX5* mutation which is associated with susceptibility to BCP-ALL in two unrelated kindreds. This mutation causes the substitution Gly183Ser in the octapeptide domain, reducing the activity of mutant PAX5 (Shah *et al.,* 2013). Overall, the majority of *PAX5* point mutations identified so far affect functional domains and result in reduced expression of *PAX5* mRNA and lost or altered DNA-binding or transcriptional regulatory activity (Mullighan *et al.*, 2007).

PAX5 amplifications are less common and include partial amplification of a subset of internal exons and complete amplification of the gene (Mullighan *et al.*, 2007; Familiades *et al.,* 2009).

Translocations of *PAX5* occur in 2-3% of pediatric BCP-ALL cases and cause the fusion of *PAX5* with several partners, thus resulting in aberrant fusion genes that preserves the correct reading frame (Mullighan *et al.*, 2007). The first case of a translocation resulting in an aberrant fusion gene involving *PAX5* was identified in 2001, when was reported the fusion of *PAX5* with *ETV6* (Cazzaniga *et al.*, 2001). Since this first report, more than 20 partner genes have been identified in several chromosomes (An *et al.,* 2008). The fusion genes can be subdivided into different groups according to the function of the PAX5 partner protein, which can be structural protein such as ELN, kinases such as JAK2, carriers of molecules, co-activator proteins, proteins involved in transcription regulation, transcription factors such as ETV6 and FOXP1 or proteins of unknown function like C20orf112. Despite several fusion genes involving *PAX5* have been identified, clues of their biological activity in leukemogenesis process have been reported only for few of them and the role of fusion genes in leukemia is totally unknown for the majority of the identified alterations (Fazio *et al.,* 2011).

Commonly, PAX5 fusion proteins result in the fusion of the N-terminal paired box DNA binding domain of PAX5 with the C-terminal region of the partner protein, whose domains substitute PAX5 regulatory domains. These feature suggests that PAX5 fusion proteins could bind to the promoter region of PAX5 target genes and act as aberrant transcription factors thus deregulating the physiological pathway of wild type PAX5. Accordingly, PAX5/ETV6, PAX5/FOXP1 and PAX5/C20orf112 fusion proteins compete with wild-type PAX5 for the PAX5 binding sequences in reporter gene assays using the murine *CD19* promoter (Kawamata *et al.,* 2008). The ability of these fusion proteins to bind to PAX5 consensus sequence has various consequences both at molecular and cellular level; in fact, PAX5 fusion proteins, *in vitro,* are able to repress the expression of several PAX5 target genes, such as *CD19, CD79a* and *BLNK,* resulting in reduced PAX5 activity and causing the block of B cell differentiation. Hence, PAX5 fusion proteins act in a dominant negative manner over endogenous PAX5 protein (Fazio *et al.,* 2008; Kawamata *et al.,* 2008). However, PAX5/ETV6 does not exclusively act in a dominant negative manner, but its role is more complex (Fazio *et al.,* 2008; Fazio *et al.,* 2013); PAX5/ETV6 activity in leukemia will be discussed later in a specific chapter.

Despite the role of PAX5 translocations has not been elucidated, there is a common feature that characterize patients carrying either a mutation, a deletion or a fusion gene, which is the significant downregulation of *PAX5* expression or reduced levels of PAX5 protein and corresponding activity (Mullighan *et al.,* 2007; An *et al.,* 2008). This evidences, in addition to the finding that some PAX5 fusion proteins act in a dominant negative manner, suggest that *PAX5* haploinsufficiency could be a mechanism of leukemogenesis. However, B cell development occur normally in heterozygous *Pax5*$^{+/-}$ mice and conditional inactivation of *Pax5* does not cause leukemia development in mice, instead, the complete loss of *PAX5* in B cells leads to an aggressive progenitor cell lymphoma (Cobaleda *et al.,* 2007b). Moreover, human individuals carrying the heterozygous inherited *PAX5* mutation 547G>A are mostly phenotypically normal or 'subnormal' in B cell development and all family members with BCP-ALL have also the concomitant deletion of the wild-type *PAX5* allele. *PAX5* 547G>A, encoding Gly183Ser, is the only inherited *PAX5* mutation identified so far and results in modest attenuation of PAX5 activity indicating that this partial hypomorphic allele is tolerated as a germline allele and additional genetic events further reducing PAX5 activity are required to establish the leukemic clone (Shah *et al.,* 2013; Auer *et al.,* 2014).

2.5. *ETV6*

2.5.1. *ETV6* gene and encoded protein structure

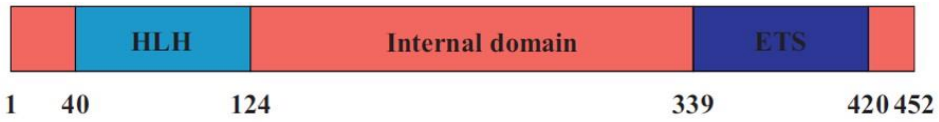

Figure 10: *ETV6* functiona domains. Helix-Loop-Helix (HLH) domain; internal domain; E-Twenty-Six (ETS) domain (Modified from De Braekeleer *et al.*, 2012).

ETV6 (*E-Twenty-Six Variant gene 6*), also known as *TEL* (*Translocation Ets Leukemia*), belongs to the *ETS* (*E-Twenty-Six*) family of transcription factor; in human it is located on chromosome 12 and has 8 exons (De Braekeleer *et al.*, 2012). Also in zebrafish *etv6* has 8 exons and it has been suggested that *etv6* may be located on chromosome 4 however, it is not mapped on a specific chromosome in the actual assembly of the genome (GRCz10/danRer10; Montpetit and Sinnet, 2001). *ETV6* gene encodes a 452aa nuclear protein that is characterized by the C-terminal ETS domain which is responsible for the binding of DNA at a purine-rich GGAA/T core motif within the promoter of the target genes (Fig. 10; Poirel *et al.*, 1997). The nuclear localization of ETV6 depends on the C-terminal region of the protein, starting from amino acidic residue 332, that includes the ETS domain and contains a putative bipartite nuclear localization signal motif (Park *et al.*, 2006). ETV6 protein is characterized also by the Helix-Loop-Helix (HLH) domain, also known as Pointed or Sterile Alpha Motif domain, located at the N-terminus of the protein (Fig. 10; Donaldson *et al.*, 1996; Bohlander, 2005). The HLH domain is involved in ETV6 homodimerization and oligomerization with other ETS proteins like Fli1 and ETV7 (Fig. 10; Kwiatkowski *et al.*, 1998; Potter *et al.*, 2000). ETV6 acts mainly as a transcriptional repressor consequently to homodimerization and heterodimerization with several repressor cofactors that are recruited by the HLH domain or the central region, also called internal domain, of ETV6 protein (Fig. 10; Lopez *et al.*, 1999; Chakrabarti and Nucifora, 1999). The internal domain of ETV6 interact with SMRT, mSin3A and N-CoR corepressors that in turn can form a repressor complex with HDACs (Chakrabarti and Nucifora, 1999; Guidez *et al.*,2000). Differently, L(3)MBT polycomb group protein interacts with the HLH domain of ETV6 enhancing its ability to repress the activation of ETV6-responsive promoters in a HDAC independent manner (Boccuni *et al.*, 2003). However, HLH domain is also involved in the recruitment of cofactors that can reduce ETV6 ability to repress transcription; in fact, HLH domain can bind to the ubiquitin-conjugating

enzyme UBC9, which positively modulates ETV6 transcriptional activity restoring the activation of a promoter that is repressed by ETV6 alone (Chakrabarti *et al.*,1999). The activity of ETS family proteins is regulated by Ras/MAP kinases through phosphorylation (Wasylyk *et al.,* 1998). ETV6 is phosphorylated at multiple sites by p38 MAP kinase *in vivo* (Poirel *et al.,* 1997; Arai *et al.,* 2002). Specifically, phosphorylation at serine 22 residue is constitutive whereas phosphorylation at serine 257 is inducible and reduces ETV6 repressive activity (Arai *et al.,* 2002). A comparative analysis performed on ETV6 genes from human to fish showed a high degree of conservation of the HLH and ETS domains with an identity of 81% and 95% respectively between human and fugu (*Takifugu rubripes*) while the overall amino acid sequence identity and similarity between the fugu and human ETV6 proteins is 58% and 72%, respectively (Montpetit and Sinnet, 2001). Moreover, the serine 22 and 257 phosphorylable residues are conserved in zebrafish (Montpetit and Sinnet, 2001).

2.5.2. *ETV6* role in embryonic development

Etv6 expression pattern has been analyzed by northern blot and *in situ* hybridization in mouse embryos and adult tissues (Wang *et al.,* 1997; Wang *et al.,* 1998). *Etv6* mRNA expression starts from E 7,0 and it markedly increases at E 17. At E 8,5 and E 9,5 *Etv6* is expressed throughout the embryo and the yolk sac whereas at E 12,5 *Etv6* mRNA is observed in several tissues and organs including cranial nerve ganglia, dorsal root ganglia, ventral region of the caudal neural tube, lung, kidney and liver; then, its expression is higher in the fetal liver and the thymus when compared with other tissues at E 14,5. *Etv6* transcript is present in several adult mouse tissues such as the brain, the heart, the kidney, the spleen and the liver as well as various cell lines representing several blood cell lineages (Fig. 11; Wang *et al.,* 1997; Wang *et al.,* 1998). It has been reported that during zebrafish embryonic development *etv6* is expressed in endothelial cells at 24hpf (Roukens *et al.,* 2010).

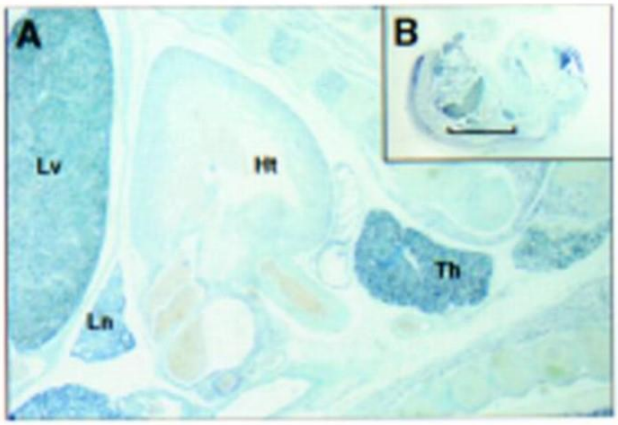

Figure 11: *Etv6* is expressed in thymus, liver and lung of mouse embryo. In situ hybridization of *Etv6* performed on E14.5 paraffin-embedded embryos. Thymus (Th); Liver (Lv); Heart (Ht); Lung (Ln) (Modified from Wang *et al.,* 1998).

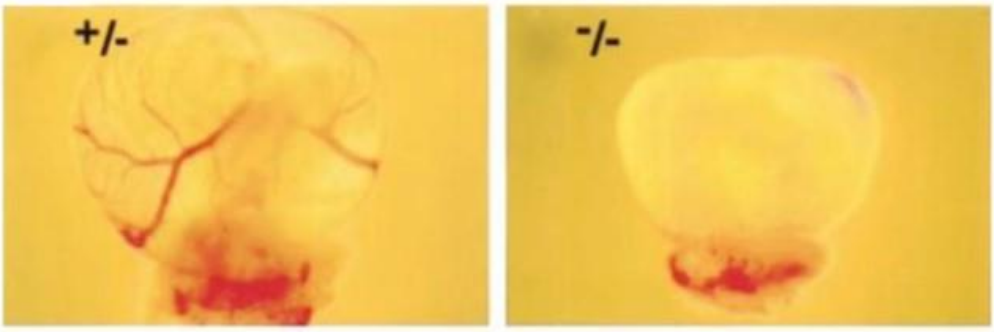

Figure 12: Yolk sac angiogenic defects in *Etv6*$^{-/-}$ embryos. E9,5 *Etv6*$^{-/-}$ embryos lack branching vitelline vessels (Modified from Wang *et al.*, 1997).

To characterize the role of *Etv6* in mammal development a knockout murine line (*Etv6*$^{-/-}$) has been generated (Wang *et al.,* 1997). *Etv6* knockout is embryonic lethal and *Etv6*$^{-/-}$ mice die between E 10,5 and E 11,5 with apoptosis of mesenchymal and neural cells and defective yolk sac angiogenesis, an alteration already observed in previous developmental stages (Fig. 12. Wang *et al.,* 1997). Accordingly, *ETV6* knockdown by means of short hairpin RNA (shRNA) expression in human umbilical vein endothelial cells (HUVECs) showed that *ETV6* is essential for the sprouting of endothelial cells, a key mechanism in the angiogenesis process (Roukens *et al.,* 2010). The role of *Etv6* in endothelial cell sprouting is conserved from mammals to fish (Roukens *et al.,* 2010). In fact, *Etv6* knockdown by morpholino microinjection in zebrafish embryos caused the alteration of intersomitic vessels formation in 70-80% of the embryos; the observed defects are: reduction in the number of vessels, aberrant vessel trajectories and the premature stalling of dorsal aorta sprouts (Fig. 13; Roukens *et al.,* 2010).

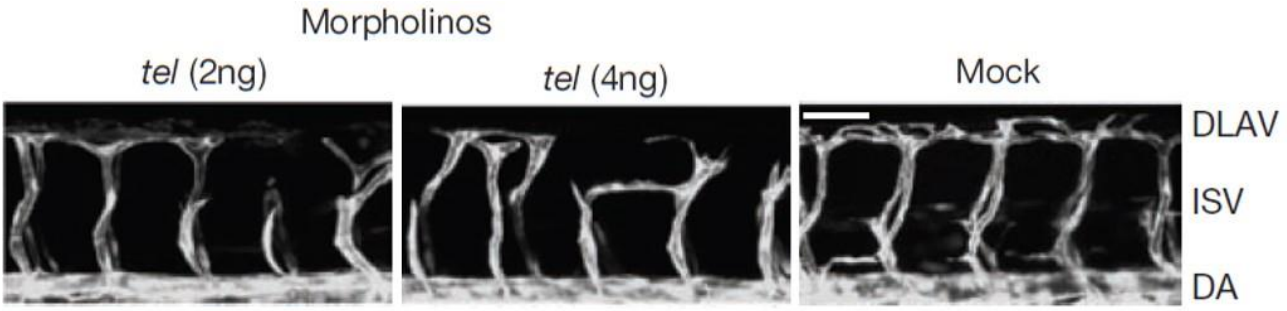

Figure 13: *etv6* (*tel*) knockdown caused the alteration of intersomitic vessels formation in zebrafish embryos. Dorsal aorta (DA); dorsal longitudinal anastomotic vessel (DLAV); intersomitic vessels (ISV) (Modified from Roukens *et al.,* 2010).

2.5.3. *ETV6* role in hematopoiesis

Consistent with its expression in murine blood cell lines, *Etv6* is involved also in hematopoiesis (Wang *et al.,* 1997; Wang *et al.,* 1998). The generation of mouse chimeras with *Etv6*$^{-/-}$ embryonic stem cells (ESCs) showed that mutant ESCs do not contribute to bone marrow myelopoiesis and erythropoiesis in chimeras (Wang *et al.,* 1998). Moreover, the conditional deletion of *Etv6* using the Cre-Lox system caused the decrease of HSCs in the bone marrow, the site of definitive hematopoiesis in adult mice, while already committed hematopoietic cells derived from this HSCs are unaffected and transiently sustain blood formation (Hock *et al.*, 2004). However, Hematopoiesis in yolk sac and fetal liver, the sites of hematopoiesis in murine fetus, occurs properly in *Etv6* null mice suggesting that in mammals *Etv6* is required for establishing hematopoiesis within the bone marrow but is dispensable for primitive and definitive embryonic hematopoiesis that occur in yolk sac and fetal liver (Wang *et al.,* 1997; Wang *et al.,* 1998; Hock *et al.,* 2004). The role of *Etv6* in hematopoiesis is not limited to the correct establishment of HSCs in the bone marrow but it is involved also in erythropoiesis (Waga *et al.,* 2003; Takahashi *et al.,* 2005; Eguchi-Ishimae *et al.,* 2009). In fact, *in vitro* experiments showed that *Etv6* expression in mouse erythroleukemia cells enhanced erythroid differentiation (Waga *et al.,* 2003). Moreover, *ETV6* overexpression in human leukemia cell line UT-7/GM, that differentiates into erythroid cells if treated with erythropoietin or megakaryocytic lineages if exposed to thrombopoietin, inhibited megakaryocytic maturation and stimulated erythroid differentiation through the stimulation of hemoglobin synthesis and upregulation of erythroid differentiation specific genes (Takahashi *et al.,* 2005). The role of *Etv6* in erythropoiesis has been confirmed also *in vivo* by the generation of a transgenic mice line expressing human *ETV6* under the control of *Gata1* promoter (Eguchi-Ishimae *et al.,* 2009). Hemoglobin concentration and red blood cell count was higher in peripheral blood of transgenic nice when compared with wild type mice while erythrocytes precursors isolated from bone marrow of transgenic mice are characterized by the upregulation of the erythroid specific genes *Alas-e* and *β-major globin* and when these cells were cultured *in vitro* with EPO expanded more efficiently with respect to controls (Eguchi-Ishimae *et al.,* 2009). Overall, *in vitro* and *in vivo* experiments suggest that *Etv6* stimulates erythroid proliferation and differentiation in mammals (Waga *et al.,* 2003; Takahashi *et al.,* 2005; Eguchi-Ishimae *et al.,* 2009).

2.6. *ETV6* in hematological malignancies

Somatic *ETV6* mutations, including point mutations, insertions and deletions, have been found in myelodysplastic syndromes, acute myeloid leukemia, BCP-ALL and T cell ALL. The majority of these mutations result in truncated proteins lacking one or more ETV6 functional domains and are unable to repress transcription acting in a dominant negative manner (Barjesteh *et al.,* 2005; Silva *et al.,* 2008; Van Vlierberghe *et al.,* 2011; Roberts *et al.,* 2014). Recently, several germline *ETV6* mutations have been identified and affected families displayed mild to moderate thrombocytopenia, some of them red cell macrocytosis, and predisposition for skin cancer, myelodysplastic syndrome, chronic myelomonocytic leukemia, colon cancer, and, most commonly, BCP-ALL. Five mutations have been identified in the ETS domain and one in the central domain of ETV6. Each of these mutations abrogates ETV6 nuclear localization, causing the loss of transcriptional repression activity exerted by ETV6. Importantly, addition of wild type ETV6 was unable to restore transcriptional repression, suggesting that ETV6 mutants acts in a dominant negative manner (Noetzli *et al.,* 2015; Topka *et al.,* 2015; Zhang *et al.,* 2015).

The short arm of chromosome 12 is frequently involved in chromosomal rearrangements in leukemia and myelodysplastic syndromes. These rearrangements include unbalanced translocations and deletions, causing the loss of genetic materials, as well as balanced translocations (Bohlander, 2005). More than half of the balanced translocations have breakpoints located in the band 12p13 (Kobayashi *et al.,* 1994). In 1994, Golub and colleagues identified a fusion of the *Platelet Derived Growth Factor Receptor Beta* gene (*PDGFRB*) located at band 5q31 to a previously unknown gene from chromosome 12 band p13, t(5;12)(q31:p13), which they called *TEL* (*Translocation Ets Leukemia*) and was later renamed to *ETV6*, in a patient with chronic myelomonocytic leukemia (Golub *et al.,* 1994). Since this first report, more than 40 chromosomal alterations causing *ETV6* translocations, deletions and inversions have been identified; among these aberrancies 28 translocations have also been characterized at the molecular level and allowed the identification of 30 *ETV6* partner genes (Fig. 14; Bohlander, 2005; De Braekeleer *et al.,* 2012). *ETV6* fusion genes can be subdivided into two groups according to the type of genes involved in the fusion: tyrosine kinases (*PDGFRB, NTRK3, ABL1, JAK2*) and transcription factors (*RUNX1, CDX2, PAX5*) (Fig. 14).

ETV6 fusions involving tyrosine kinases are correlated with a wide spectrum of hematological malignancies which include chronic myelomonocytic leukemia, acute myeloid leukemia, BCP-ALL, T-cell ALL, chronic myeloproliferative neoplasm, myelodysplastic syndromes, T cell lymphoma and even with solid cancers (Golub *et al.,* 1994; Golub *et al.,* 1996; Lacronique et al., 1997; Peeters *et al.,*

1997; Knezevich *et al.*, 1998; Eguchi *et al.*, 1999; Euhus *et al.*, 2002; Kralik *et al.*, 2011; De Braekeleer *et al.*, 2011; De Braekeleer *et al.*, 2012).

Usually, ETV6/tyrosine kinases fusion proteins are characterized by the C-terminal region of ETV6 containing the HLH dimerization domain and the N-terminal region of tyrosine kinases containing at least the kinase domain. The mechanism of leukemogenesis of these aberrant proteins depends on the HLH domain of ETV6, that causes the homodimerization of the fusion protein leading to the constitutive activation of the tyrosine kinase domain (Carroll *et al.*, 1996; Golub *et al.*,1996; Lacronique *et al.*,1997). The ETV6/PDGFRB, ETV6/ABL1 and ETV6/JAK2 fusions have the potential to induce leukemic transformation *in vivo*, in fact, in a murine bone marrow transplant model they caused myeloproliferative syndrome (ABL and PDGFRB), fatal myeloid and lymphoproliferative diseases (JAK2) (Schwaller *et al.*, 1998; Tomasson *et al.*, 2000; Million *et al.*, 2002). The mechanism underlying leukemic transformation induced by ETV6 fusions with tyrosine kinases has been explained, at least in part, through the identification of the targets of aberrant kinases. In fact, ETV6/PDGFRB, ETV6/ABL1 and ETV6/JAK2 are able to phosphorylate and activate STAT proteins (Wilbanks *et al.*, 2000; Spiekermann *et al.*, 2002), which are essential for the production of mature hematopoietic cells via effects on cellular proliferation, survival and lineage-specific differentiation (Dorritie *et al.*, 2014).

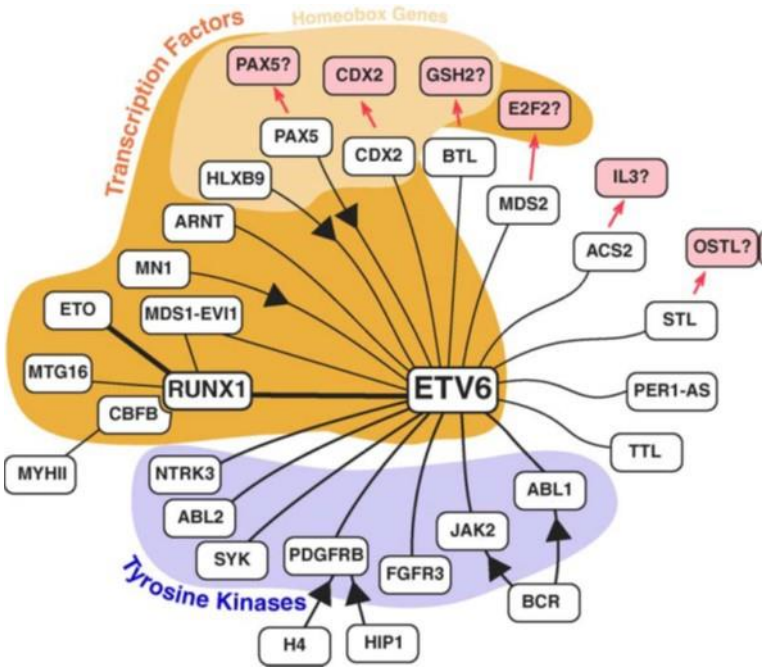

Figure 14: Fusion gene network of *ETV6*. Transcriptional upregulation is indicated by a red arrow. A question marks denotes that this upregulation or the relevance of the upregulation has not been proven (Bohlander 2005).

ETV6 fusions with other transcription factors have been associated mainly with BCP-ALL, myelodysplastic syndromes, acute myeloid leukemia, chronic myeloid leukemia and myeloproliferative disorders (Bohlander, 2005; De Braekeleer *et al.,* 2012).

The translocations that causes the fusion o *ETV6* with another transcription factor can alter the activity of one or both the partners in three ways: the *ETV6* gene provide the promotor but any functional domain (*ETV6/CDX2*), the *ETV6* gene provides the HLH domain to a partner gene in which the functional domains are retained (*ETV6/RUNX1*), ETV6 provides the HLH and ETS domains to a partner gene that is structurally modified by the fusion (*PAX5/ETV6*) (De Braekeleer *et al.,* 2012).

ETV6 does not contribute with functional domains in *ETV6/CDX2* aberrant gene and it is likely that its promoter drives the transcription of *CDX2* (Chase *et al.,* 1999; Rawat *et al.,* 2004).

ETV6/RUNX1 is caused by the translocation t(12;21)(p13;q22) and is the most common rearrangement in pediatric BCP-ALL, accounting for about 22% of the cases. The fusion protein retains the HLH domain and the central repression domain of ETV6 as well as the DNA binding domain and the transcription activation domain of RUNX1 (Shurtleff *et al.,* 1995; Mullighan, 2012). The ETV6/RUNX1 fusion protein retains the ability to bind the RUNX1 target sequences, but represses the expression of genes activated by RUNX1 (Hiebert *et al.,* 1996; Guidez *et al.,* 2000). Expression of ETV6/RUNX1 promotes self-renewal in B cell progenitors but alone does not induce leukemia (Andreasson *et al.,* 2001; Morrow *et al.,* 2004). In fact, the expression of *ETV6/RUNX1* under the control of the immunoglobulin heavy chain promoter did not cause leukemia in a transgenic mouse model suggesting that secondary genetic events are required to induce leukemia (Andreasson *et al.,* 2001). This hypothesis is supported by the identification of additional recurring genetic alterations in *ETV6/RUNX1* ALL, including deletions of *PAX5*, *EBF1* and deletion of the second copy of *ETV6* (Mullighan *et al.,* 2007; Mullighan, 2012).

2.7. *PAX5/ETV6*

PAX5/ETV6 fusion gene has been described in 2001 in a patient with ALL. This aberrant gene results from the translocation t(9;12)(q11;p13) with the breakpoints located in intron 4 of *PAX5* and intron 2 of *ETV6.* This translocation did not cause the alteration of the reading frame, thus, the fusion gene encodes an aberrant protein that retains the paired box DNA binding domain of PAX5 and all ETV6 functional domains, which are the HLH, the central and the ETS domains (Fig. 15; Cazzaniga *et al.,*

2001). The translocation resulting in *PAX5/ETV6* is the most common among *PAX5* translocations (Mullighan *et al.,* 2007; An *et al.,* 2008; Kawamata *et al.*,2008).

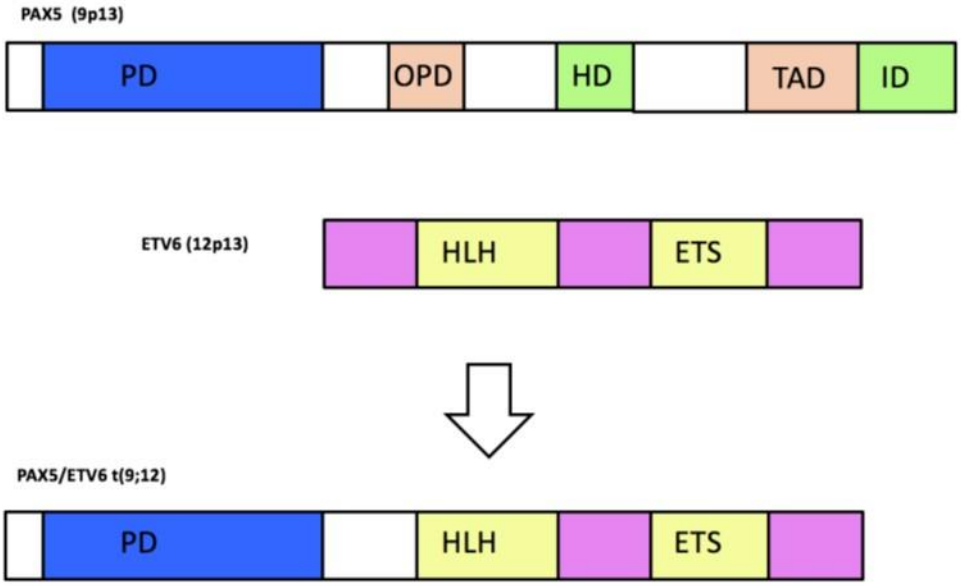

Figure 15: Schematic representation of PAX5, ETV6 and the resulting PAX5/ETV6 fusion protein. partial homeodomain (HD); inhibitory domain (ID); octapeptide domain (OPD); paired box domain (PD); transactivation domain (TAD); Helix-Loop-Helix (HLH) domain; internal domain; E-Twenty-Six (ETS) domain.

It has been suggested that PAX5/ETV6 exerts a dominant negative activity on endogenous PAX5 because it is able to interact with the PAX5-consensus sequence contained in the *CD19* promoter, competing with wild type protein (Kawamata *et al.,* 2008; Kawamata *et al.,* 2012). Accordingly, the transfection of *PAX5/ETV6* in murine pre-BI cell precursors derived from fetal liver caused the downregulation of several genes whose expression is activated by PAX5 such as *CD19*, *BLNK* and *CD79a* (Fazio *et al.*,2008). The conversion of PAX5 in a repressor seems to be mediated by the functional domains of ETV6, in fact, PAX5/ETV6 co-immunoprecipitates with the ETV6 co-repressor mSin3a. However, PAX5/ETV6 activity is not strictly limited to a dominant negative effect, because gene expression profiling analysis showed that PAX5/ETV6 is able to down-regulate the expression of some PAX5-activated-genes, such as *Cd79a* and *Blnk*, as well as to up-regulate some PAX5-repressed-genes, such as *Lck* (Fazio *et al.,* 2013). Thus, it has been proposed that *PAX5/ETV6* act in an "opposite dominant" manner instead of a canonical dominant negative effect. Interestingly, PAX5/ETV6 expression caused the reduction of endogenous Pax5 both at protein and at mRNA

levels, suggesting that PAX5/ETV6 activity, together with the loss of one allele of wild type *PAX5* that occur in translocated patients, could determines a PAX5 haploinsufficiency situation (Fazio *et al.,* 2013).

PAX5/ETV6 has been transfected in murine pre-BI cell precursors derived from fetal liver to study *in vitro* its role in the pathogenesis of leukemia. FACS analysis showed that cells transfected with *PAX5/ETV6* downregulated B220 and CD19 proteins, which are B cell precursors specific antigens. Moreover, after the stimulation of B cell differentiation, control cells started to express μ heavy chain whereas *PAX5/ETV6* expressing cells did not, thereby highlighting the impairment of pre-BCR assembly and consequently the block of B cell differentiation (Fazio *et al.,* 2008).

PAX5/ETV6 confers also novel features to the expressing cells, such as an increased migration ability and survival advantages. In fact, *PAX5/ETV6* enhanced cell migration ability toward CXCL12 chemokine. Accordingly, it has been observed the upregulation of *CXCR4*, encoding a chemokine receptor involved in hematological malignancies (Burger and Burkle, 2007), in *PAX5/ETV6* expressing cells. Interleukin 7 is necessary for *in vitro* pre-BI cell survival and, although *PAX5/ETV6* does not confer IL-7 independence to pre-BI cells for prolonged periods, it gives a survival advantage under IL-7 starvation conditions. Moreover, pre-BI cells expressing *PAX5/ETV6* grew in the presence of TGFβ1, a cytokine with antiproliferative and proapoptotic effects, showed increased proliferation and survival compared with control.

The acquisition of these survival advantages in PAX5/ETV6 expressing cells is due, at least in part, to the activation of the Lck-STAT5 pathway (Fig. 16; Cazzaniga *et al.*, 2015). In fact, PAX5/ETV6 expression in murine cells caused the overexpression of *Lck* mRNA and the activation of Lck protein through the de-phosphorylation of the Lck inhibitory domain Y505. In turn, Lck causes the phosphorylation of STAT5 at the activator residue Y694 and consequently STAT5 activation leading to the overexpression of STAT5 effectors *cMyc* and *Ccnd2*. Accordingly, the treatment with the Lck activity inhibitor BIBF1120 caused a significant reduction of $Stat5^{Y694}$ phosphorylation and reduced the replicative rate of PAX5/ETV6 expressing cells, thus abrogating their proliferative advantage. Interestingly, the increased LCK and STAT5 phosphorylation was observed also in cells from *PAX5* translocated patients and also in these cells the treatment with BIBF1120 caused the reduction of STAT5 phosphorylation (Fig. 16; Cazzaniga *et al.*, 2015).

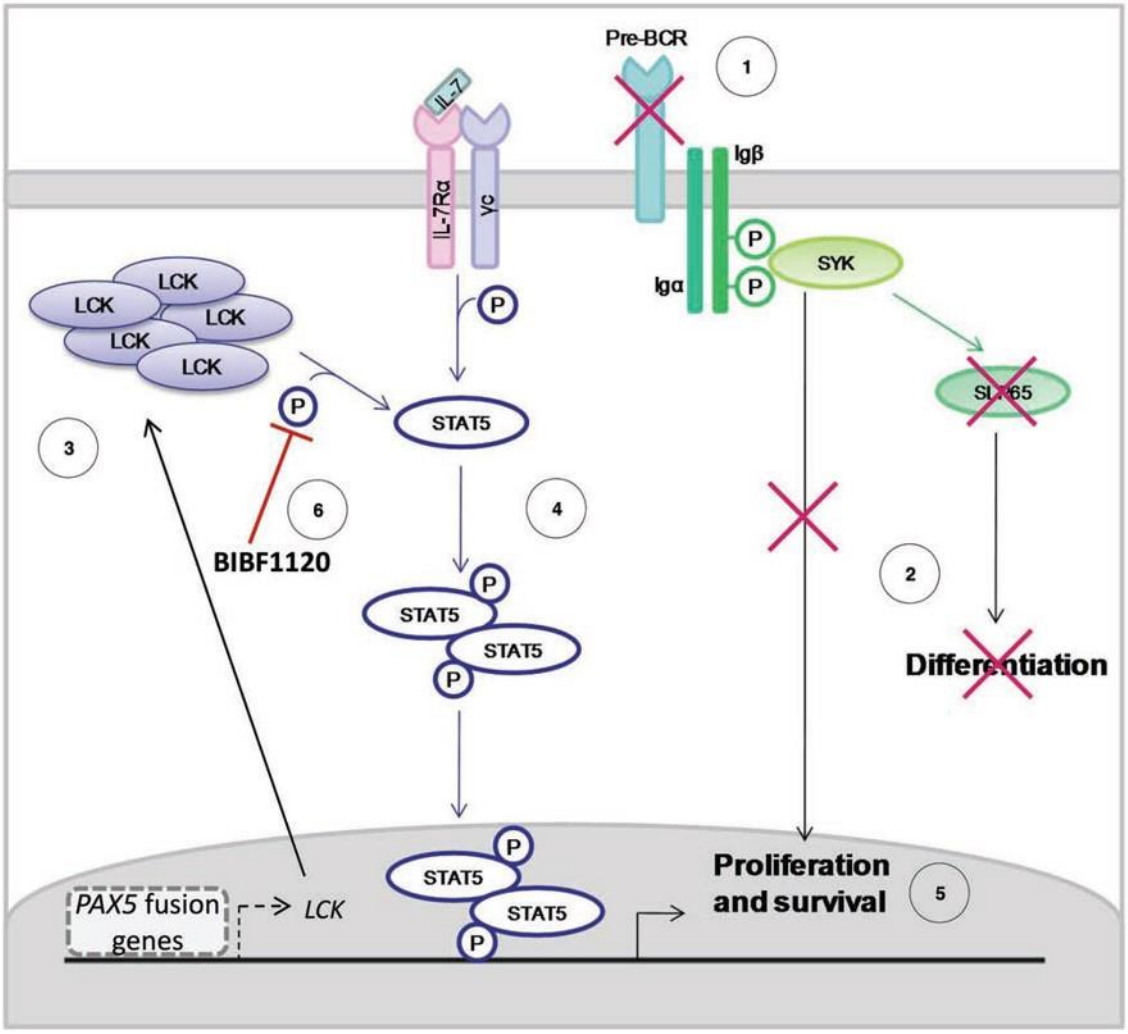

Figure 16: Mechanism of action of PAX5/ETV6 fusion protein in BCP-ALL. The pre-BCR is not expressed in PAX5/ETV6 expresing cells (A), causing the typical proliferation advantage and differentiation block of BCP-ALL (B). In particular, PAX5/ETV6 cause the up-regulation of LCK and its activation (C). LCK activation leads to STAT5 phosphorylation (D), thus sustaining proliferation and survival of blast cells via the transcription of STAT5 target genes (E). The administration of the LCK inhibitor BIBF1120 reverts this phenomenon, inhibiting STAT5 signaling (F) (Cazzaniga *et al.*, 2015).

3. Aim of the Project

The current knowledge about the role of *PAX5/ETV6* fusion gene in the pathogenesis of leukemia derive only from *in vitro* studies and it is necessary to establish an *in vivo* model system which could be useful in future to gain new insights into the role of this aberrant gene in leukemia and to perform drug screenings.

We are performing this project in collaboration with the group led by Dr. Giovanni Cazzaniga and Prof. Andrea Biondi which is currently studying the role of this aberrant gene in leukemogenesis *in vitro*, at the Tettamanti Foundation research center (San Gerardo hospital, University of Milano-Bicocca, Monza).

When we started this project there was no published information about the involvement of *pax5* and *etv6* in zebrafish hematopoiesis. Hence, the first issue to resolve was to verify the conservation of the roles of these genes in hematopoiesis during zebrafish development. To accomplish this task, we first analyzed the expression pattern of *pax5* and *etv6* by whole mount hybridization assays to verify if they are expressed in hematopoietic tissues during zebrafish development. Then, we performed loss of function experiments through the injection of morpholino anti-sense oligonucleotides for both *pax5* and *etv6*. In *pax5* knockdown embryos, we analyzed the expression of several B cell markers to confirm that *pax5* is involved in B cell lymphopoiesis also in zebrafish. Since it is known that *etv6* is involved in erythrocytes differentiation, we tested the effect of *etv6* loss of function on primitive erythropoiesis during zebrafish development and we tried to shed light on the potential molecular pathways which involve *etv6* in this process.

The final aim of this project is to verify if zebrafish could be a suitable model to study BCP-ALL related to *PAX5/ETV6* fusion gene. To this end, we injected *PAX5/ETV6* mRNA in zebrafish embryos and we tested if the expression of this aberrant gene is able to induce the same molecular alteration observed by *in vitro* experiments, such as the alteration of the expression of B cell markers and the activation of Lck-STAT5 pathway (Fazio *et al.*,2008; Fazio *et al.*,2013; Cazzaniga *et al.*, 2015).

It has been proposed that the loss of one wild type *PAX5* allele and downregulation of *PAX5* expression or reduced levels of PAX5 protein in patients carrying either a mutation, a deletion or a fusion gene could be a mechanism of leukemogenesis (Mullighan *et al.,* 2007; An *et al.,* 2008). However, additional genetic events further reducing PAX5 activity are required to establish the leukemic clone (Cobaleda *et al.,* 2007b; Mullighan *et al.,* 2007; Shah *et al.,* 2013; Auer *et al.,* 2014). Since BCP-ALL patients carrying *PAX5-ETV6* are also characterized by the mono-allelic losses of

PAX5 and *ETV6*, we decided to test if the contemporary *pax5* and *etv6* knock-down could cooperate to alter the expression of B cell markers.

4. Main Results

4.1. *pax5* is expressed in pancreas of zebrafish larvae and its knockdown alters the expression of B cell markers

In order to verify that *pax5* is expressed in organs involved in hematopoiesis during zebrafish development, we performed whole mount *in situ* hybridization (WISH) from 24 hpf to 5 dpf. In addition to the previously reported tissues (Pfeffer *et al.,* 1998; Kwak *et al.,* 2006; data not shown), we observed a weak staining of the *pax5* probe in the gut and hybridization signals in liver and in pancreas, which has been proposed as a transient site of B cell development in zebrafish (Danilova and Steiner, 2002), from about 60 hpf to 5 dpf. Since it is not known if the role of *pax5* in B cell development is conserved in zebrafish, we analyzed if *pax5* knockdown causes the alteration of B cell markers expression at 5 dpf. The injection of a *pax5* translation blocking morpholino (pax5-MO) caused the downregulation of *igh*μ and *cd79a*, as well as the upregulation of *cd79b* compared to control embryos, as showed by qRT-PCR assays. These findings suggest that the role of *pax5* in B cell development could be, at least in part, conserved in zebrafish.

4.2. *etv6* is involved in primitive erythropoiesis in zebrafish

We characterized the *etv6* expression pattern by WISH assays and we found that *etv6* is expressed in several hematopoietic tissues. Indeed, *etv6* is expressed in the ALM and in the PLM at the 10 somite stage. Then, *etv6* transcript is present in the ICM at the 20 somite and 24 hpf stages. At 36 hpf *etv6* is expressed in the caudal hematopoietic tissue (CHT), which a transient site of definitive hematopoiesis (Chen and Zon, 2009), and in the ventral wall of the dorsal aorta, that correspond to the Aorta-Gonad-Mesonephros (AGM). Besides hematopoietic tissues, *etv6* is expressed also in the nervous system.

To investigate the role of *etv6* in hematopoiesis, we performed *etv6* knockdown by the injection of a translation blocking morpholino that it has been already published and validated (Roukens *et al.,* 2010). However, we verified that *etv6*-MO causes the nonspecific activation of p53 (Robu *et al.,* 2007). Consequently, we decided to coinject *etv6*-MO and *p53*-MO in the subsequent experiments to suppress off-target effects and facilitate the study of specific loss of function phenotypes.

Since *etv6* is involved in erythropoiesis in mammals and we found that it is expressed in tissues related to primitive erythropoiesis (Waga *et al.,* 2003; Takahashi *et al.,* 2005; Eguchi-Ishimae *et al.,* 2009), we decided to verify if *etv6* is involved in primitive erythropoiesis in zebrafish. Thus, we

analyzed the hemoglobin content of *etv6* morphants by o-dianisidine staining and we found that primitive mature erythrocytes were reduced in *etv6*-MO/*p53*-MO coinjected embryos at 48 hpf. However, the production of primitive erythrocytes precursors is not impaired in *etv6* morphants because we did not observe a decrease of circulating blood cells at 48 hpf and *etv6* knockdown did not cause the alteration of the expression of primitive erythrocytes markers (*hbbe1*, *hbbe3*, *gata1*) at 24hpf, when differentiation of primitive erythrocytes is about to start. So, we analyzed the expression of *hbbe3* and *gata1* at 48 hpf when these genes must be downregulated to allow primitive erythrocytes differentiation and we found that they are still up-regulated in *etv6* morphant embryos suggesting that *etv6* knock-down caused the alteration of primitive erythrocytes maturation.

The increased activation of Notch pathway signaling and the consequent upregulation of the downstream target *her6* can cause the alteration of primitive erythrocytes differentiation (Bresciani *et al.,* 2010). Hence, we analyzed *her6* expression by WISH assays and we found that it is upregulated and expanded in the PLM of *etv6* morphants at 10 somites. Accordingly, the treatment with the Notch inhibitor DAPT partially rescued the alteration of primitive erythrocytes differentiation in *etv6* morphants at 48 hpf. Thus, the defective primitive erythrocytes maturation in *etv6* morphants is, at least in part, caused by the increased activity of the Notch pathway.

Recently, it has been reported that *ETV6* mutations can alter the expression of *KLF6* (Zhang *et al.,* 2015). The zebrafish *klf6a* gene, along with *klf1, klf3* and *klf17* is involved in the differentiation of primitive erythrocytes (Gardiner *et al.,* 2007; Fu *et al.,* 2009; Xue *et al.,* 2015), we thus decided to test by q RT-PCR analysis if *etv6* knockdown can cause the alteration of these genes. *etv6* knockdown induced the statistically significant upregulation of *klf3* and *klf17* at 24 hpf as well as of *klf1, klf3, klf6a* and *klf17* at 48 hpf.

A recent report showed that *etv6* knockdown caused the alteration of lymphoid precursors markers *rag1* and *ikaros* (Rasighaemi *et al.,* 2015). Hence, we tested if *etv6* loss of function could induces the deregulation of B cell markers. Using qRT-PCR analysis we observed that *etv6* knockdown caused the downregulation of *igh*μ, *cd79a* and *blnk,* whereas it induced the upregulation of *cd79b*.

4.3. The injection of *PAX5/ETV6* mRNA induced the activation of Lck-Stat5 pathway but did not caused the alteration of B cell markers expression

To verify if zebrafish could be a suitable model to study the role of *PAX5/ETV6* fusion gene in BCP-ALL, we transiently expressed the human *PAX5/ETV6* gene in zebrafish embryos by the injection of

two doses of its mRNA. However, the expression of *cd79a* and *blnk* was not statistically significant altered in embryos injected with *PAX5/ETV6* mRNA at 5 dpf, while they are downregulated in B cell precursors *in vitro* (Fazio *et al.,* 2008).

PAX5/ETV6 caused the upregulation of *Lck* mRNA and enhanced Lck kinase activity *in vitro* (Cazzaniga *et al.,* 2015). Thus, we analyzed the expression of *lck* by WISH assays at 5 dpf and we found that it is upregulated and expanded in the thymus of embryos injected with *PAX5/ETV6* mRNA.

Lck kinase activity is enhanced by phosphorylation of the tyrosine Y394 (Hui and Vale, 2014). To verify if PAX5/ETV6 causes the activation of Lck protein also in zebrafish, we performed western blot assays using an anti-Lck^{Y394} antibody and we found that the level of Lck phosphorylated protein is increased in embryos injected with *PAX5/ETV6* mRNA in a dose dependent manner.

The activation of Lck protein induced by PAX5/ETV6 in murine pre-BI cells is responsible for the phosphorylation of the STAT5 tyrosine residue 694, leading to the activation of the protein and the upregulation of the STAT5 effectors *cMyc* and *Ccnd2* (Cazzaniga *et al.,* 2015). Western blot assays performed at 5 dpf using an anti-STAT5^{Y694} showed that the injection of 250 pg/embryo of *PAX5-ETV6* mRNA strongly increased the level of STAT5^{Y694} phosphorylated protein in zebrafish embryos, whereas the injection of 200 pg/embryo of *PAX5/ETV6* mRNA did not.

Since the homologues of the murine STAT5 effectors *cMyc* and *Ccnd2* are duplicated in zebrafish, we analyzed the expression of *cmyca*, *cmycb* and *ccnd2a* genes by qRT-PCR assays at 5dpf in embryos injected with *PAX5/ETV6* mRNA. The expression of all the Stat5 effectors analyzed is statistically significant upregulated at both doses of *PAX5/ETV6* mRNA showing a dose dependent effect.

Thus, PAX5/ETV6 expression in zebrafish embryos is able to induce the phosphorylation and activation of Lck and Stat5, as well as the upregulation of the Stat5 effectors *cmyca*, *cmycb* and *ccnd2a*.

4.4. *pax5* and *etv6* do not cooperate in the regulation of *igh*μ and *cd79a* expression

Since *etv6* knockdown caused the alteration of the expression of *igh*μ and *cd79a* which are impaired also in *pax5* morphants, we decided to investigate if the contemporary *pax5* and *etv6* knock-down could cooperate to alter their expression. To this end we coinjected lower doses of both *pax5* and *etv6* morpholinos. Using these doses, we observed the downregulation of *igh*μ in both *pax5* and *etv6* single morphants, and *cd79a* in *etv6* single morphants, however these variations are not statistically

significant when compared with control embryos. The coinjection of *pax5* and *etv6* morpholinos did not cause significant alteration of *igh*μ and *cd79a* expression if compared with single morphants or control embryos. Hence, these experiments seem to exclude a possible cooperation between *pax5* and *etv6* in the regulation of *igh*μ and *cd79a* expression.

5. Conclusions

Zebrafish has proven to be a useful model to study physiological hematopoietic processes as well as pathological alterations involved in human hematological malignancies (Lieschke and Currie, 2007; Paik and Zon, 2010; Jing and Zon, 2011). However, despite many efforts have been made to deepen the knowledge of zebrafish hematopoiesis, very little is known about zebrafish B cell development. Our findings about *pax5* expression in the pancreas of zebrafish embryos support the hypothesis proposed by Danilova and Steiner (2002) that the pancreas could be a transient site of B cell development in this teleost fish (Danilova and Steiner, 2002).

In mammals, *PAX5* is essential for B cell commitment and for the maintenance of B cell identity throughout B cell maturation (Cobaleda *et al.,* 2007). Overall, our genetic evidences suggest that *pax5* role in B cell development could be, at least in part, conserved in zebrafish because *pax5* loss of function determined the alteration of the expression of several components of the BCR such as *cd79b, igh*μ and *cd79a*. Since B cell emergence is a late event during zebrafish development, it will be necessary to establish *pax5* mutant lines in order to study B cell phenotype in larval stages and adult fish to better characterize the role of *pax5* in zebrafish B cell development.

ETV6 accelerates erythroid differentiation of human and mouse leukemia cell lines and it stimulates hemoglobin synthesis in adult mice (Waga *et al.,* 2003; Takahashi *et al.,* 2005; Eguchi-Ishimae *et al.,* 2009). Accordingly, we found that *etv6* is essential for primitive erythrocytes maturation. Morover, we tried to shed light into the mechanism through which *etv6* regulates primitive erythrocytes maturation as a potential modulator of the notch signaling in primitive erythrocytes differentiation. Furthermore, *etv6* could potentially influences several processes of erythrocytes differentiation via the modulation of *klf* genes expression. In fact, we show here that *etv6* is involved, directly or indirectly, in the repression of several *klf* genes, which are essential for primitive erythrocytes maturation through the activation of multiple erythroid-specific genes (Palis, 2014). Thus, our findings suggest that *etv6* role in hematopoiesis could be conserved from fish to mammals. However, we observed the alteration of primitive erythropoiesis whereas in mouse *Etv6* is necessary only for adult hematopoiesis (Wang *et al.,* 1997; Wang *et al.,* 1998; Hock *et al.,* 2004).

The final aim of this study was to test if the expression of the human *PAX5/ETV6* fusion gene in zebrafish embryos is able to induce the same molecular alterations observed *in vitro* (Fazio *et al.,* 2008; Fazio *et al.,*2013; Cazzaniga *et al.,* 2015). PAX5/ETV6 expression confers survival advantages to pre-BI cells due to the activation of Lck protein, that in turn induces the phosphorylation and activation of STAT5 leading to the overexpression of STAT5 effectors *cMyc* and *Ccnd2* (Cazzaniga

et al., 2015). Interestingly, we found that the amount of lck protein phosphorylated in the activator residue Y394 was increased in *PAX5/ETV6* mRNA injected embryos in a dose dependent manner, thus indicating that PAX5/ETV6 can induce the activation of lck protein in zebrafish. Importantly, the zebrafish homologues of the murine STAT5 effectors *cMyc* and *Ccnd2* are upregulated in a dose dependent manner in embryos injected with *PAX5/ETV6* mRNA, suggesting that PAX5/ETV6 expression activates the same pathways involved in the acquisition of the survival advantages showed by murine pre-BI cells. However, the level of Stat5 activated protein is increased only in embryos injected with the higher dose of the *PAX5/ETV6* mRNA. It is conceivable that at the lower dose of *PAX5/ETV6* mRNA the upregulation of *cmyca*, *cmycb* and *ccnd2a* could be induced by the activation of other unknown pathways.

Overall, our results indicate that the molecular alterations caused by *PAX5/ETV6 in vitro* can be recapitulated in zebrafish, suggesting that zebrafish could be a good model system to study *in vivo* BCP-ALL associated to *PAX5/ETV6*. It will be useful to establish an inducible transgenic line to stably express the fusion gene also in later developmental stages and in adult fish to verify if longer expression of *PAX5/ETV6* can induce the development of overt leukemia. To this end, we will establish a Gal4-UAS inducible transgenic line and we have already cloned the *PAX5/ETV6* coding sequence in a vector used to generate transgenic fish through the tol2 transposon system (Kawakami, 2007; Asakawa and Kawakami, 2008).

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Part II

Zebrafish as a model to recapitulate the molecular alterations caused by *PAX5/ETV6* fusion gene in Acute Lymphoblastic Leukemia

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Abstract

The PAX5 transcription factor is essential for B cell lineage commitment and differentiation. *PAX5* is target of alterations in about 30% of B cell precursors Acute Lymphoblastic Leukemia and the most recurrent among *PAX5* translocations determines the generation of *PAX5/ETV6* fusion gene. PAX5/ETV6 caused the alteration of B cell differentiation and conferred survival advantages *in vitro* due to the activation of the Lck-STAT5 pathway. The information currently available on *PAX5/ETV6* derive mainly from *in vitro* models, we thus tested whether zebrafish could be a good model to study this aberrant gene *in vivo*. To verify that the role of *pax5* in B cell development is conserved in zebrafish, we analyzed its expression pattern and we found that it is expressed in pancreas, which has been proposed to be a transient embryonic site of B cell lymphopoiesis in zebrafish. Moreover, *pax5* knockdown caused the alteration of the expression of several B cell markers, suggesting that *pax5* role in B cell development could be conserved in zebrafish. Interestingly, *etv6* knockdown caused the alteration of the expression of some B cell markers which are impaired also by *pax5* loss of function, thus, we investigated if the contemporary *pax5* and *etv6* knock-down could cooperate to alter the expression of these genes. However, the coinjection of *pax5* and *etv6* morpholinos did not cause significant alteration of *ighμ* and *cd79a*, thus excluding a possible cooperation between *pax5* and *etv6* in the regulation of *ighμ* and *cd79a* expression. Finally, we transiently expressed *PAX5/ETV6* in zebrafish embryos and, despite it did not caused the deregulation of B cell markers expression, this aberrant gene is able to activate the same pathway which is altered *in vitro*, inducing the phosphorylation of Lck and Stat5 proteins as well as the upregulation of Stat5 effectors, suggesting that zebrafish could be a suitable model to study BCP-ALL related to *PAX5/ETV6* fusion gene.

Introduction

PAX5 (*Paired box domain* 5) belongs to the Paired Box family of transcription factors and the defining feature of PAX5 protein is the Paired Box DNA binding domain (PD; Czerny *et al.,* 1993; Cobaleda *et al.,* 2007). Transactivation activity of PAX5 is mediated by the partial Homeodomain (HD) and the Transactivation domain (TAD), however, PAX5 can be converted into a transcriptional repressor by the Octapeptide (OP) domain and the Inhibitory domain (ID; Dorlfler and Busslinger, 1996; Eberhard and Busslinger, 1999; Eberhard *et al.,* 2000; Emelyanov *et al.,* 2002; Barlev *et al.,* 2003; Cobaleda *et al.,* 2007). *PAX5* is extensively studied in mammals for its essential role in B cell commitment and maturation. *PAX5* fulfills its function mainly through the activation of B lineage-specific genes and the repression of inappropriate ones (Cobaleda *et al.,* 2007). Specifically, PAX5 activates the expression of *CD79a* and *BLNK*, whereas repress *LCK* (Schebesta *et al.,* 2002; Maier *et al.,* 2003; Cobaleda, 2007). All the functional domain of human PAX5 are highly conserved in zebrafish (Pfeffer *et al.,* 1998). During zebrafish embryonic development, *pax5* is expressed in the midbrain-hindbrain boundary and in the anterior part of the otic vesicle where it is implicated in the development of the utricular macula and it is essential for vestibular function (Pfeffer *et al.,* 1998; Kwak, 2006). However, there are no published data that confirm the involvement of *pax5* in B cell development also in zebrafish.

PAX5 is a frequent target of alterations in B cell precursors acute lymphoblastic leukemia (BCP-ALL). In fact, about 30% of pediatric patients affected by BCP-ALL are characterized by mutations, deletions or translocations of the *PAX5* gene (Mullighan *et al.,* 2007). Translocations of *PAX5* occur in 2-3% of pediatric BCP-ALL cases and cause the fusion of *PAX5* with several partner genes encoding proteins with different functions, such as structural proteins, kinases, transcription factors and others (Mullighan *et al.,* 2007; Nebral *et al.,* 2009). The most recurrent *PAX5* translocation is t(9;12), which leads to the generation of *PAX5/ETV6* fusion gene and the mono-allelic losses of each wild type gene (Cazzaniga *et al.,* 2001; Mullighan *et al.,* 2007; An *et al.,* 2008). The fusion gene encodes an aberrant protein that retains the paired box DNA binding domain of PAX5, while PAX5 regulatory domains are substituted by ETV6 functional domains, which are the Helix-Loop-Helix, the central and the ETS domains (Cazzaniga *et al.,* 2001). PAX5/ETV6 protein localizes in the nucleus and acts as an aberrant transcription factor altering the expression profile of murine B cell precursors *in vitro* (Cazzaniga *et al.,* 2001; Fazio *et al.,*2013). PAX5/ETV6 is able to down-regulate the expression of some PAX5-activated-genes, such as *Cd79a* and *Blnk*, as well as to up-regulate some PAX5-repressed-genes, such as *Lck*, in a dominant manner *versus* the endogenous PAX5 (Fazio *et al.,* 2008; Fazio *et al.,*2013). Among the genes whose expression is altered by PAX5/ETV6, there

are several ones involved in pre-BCR assembly and signaling, included *Cd79a* and *Blnk*. These alterations, together with the decreased expression of both cytoplasmic and transmembrane μ heavy chain, lead to the impairment of pre-BCR assembly, that is essential for pre-BI cells survival, proliferation and differentiation (Fazio *et al.,* 2008; Fazio *et al.,*2013). Accordingly, *PAX5/ETV6* caused the block of B cell differentiation and it also conferred novel features to the expressing cells, such as an increased migration ability and survival advantages (Fazio *et al.,* 2008). The acquisition of these survival advantages in PAX5/ETV6 expressing cells is due to the activation of the Lck-STAT5 pathway (Cazzaniga *et al.,* 2015). PAX5/ETV6 expression in murine cells caused the activation of Lck protein. In turn, Lck induces the phosphorylation and activation of STAT5 leading to the overexpression of STAT5 effectors *cMyc* and *Ccnd2*. These molecular alterations are reverted upon the treatment with the Lck activity inhibitor BIBF1120, that also reduced the replicative rate of PAX5/ETV6 expressing cells, thus abrogating their proliferative advantage (Cazzaniga *et al.,* 2015).
Zebrafish has emerged as an excellent model organism because it allows to link genetic and molecular studies to morphological whole-organism analysis and it offers several advantages compared to other vertebrate model systems. The external fertilization and development, together with the optical clarity of the embryos allow *in vivo* and *ex vivo* easy visualization of embryonic development and manipulation of embryos. Zebrafish resulted to be particularly suitable for the study of hematopoiesis and hematopoietic disorders affecting humans, because the developmental processes and genetic programs of hematopoiesis, as well as the main blood cell types, are highly conserved (Lieschke and Currie, 2007; Paik and Zon, 2010; Jing and Zon, 2011). Despite zebrafish hematopoiesis has been extensively studied, very little is known about B-cell lymphopoiesis during zebrafish development, in fact, only Danilova and Steiner (2002) suggested that the pancreas could be an embryonic site of B cell development in zebrafish. This hypothesis is based on the expression of *rag1* at 4 dpf (days post fertilization) and *ighμ* from 10 dpf in the pancreas. Subsequently, B cell development moves to the kidney at about 2 weeks post fertilization (wpf), when *rag1* starts to be expressed in this organ, while *ighμ* expression has been observed in the kidney at 19 dpf (Danilova and Steiner, 2002; Zapata *et al.,* 2006).
Zebrafish models of human leukemia caused by *ETV6/RUNX1* and *ETV6/JAK2* gene fusions have already been generated and they showed histological and molecular alterations referable to BCP-ALL and myeloid malignancies respectively. These findings suggest that zebrafish could be a useful model to reproduce and study the leukemic process *in vivo* (Onnebo *et al.,* 2005; Sabaawy *et al.,* 2006).
Herein, we show that *pax5* is expressed in tissues related to B cell lymphopoiesis during zebrafish development and that *pax5* knockdown by means of morpholino microinjection caused the alteration

of the expression of several B cell markers, thus suggesting that *pax5* is involved in in B cell development also in zebrafish. Moreover, we found that *etv6* knockdown caused the alteration of some B cell markers which is impaired also by *pax5* loss of function. This finding prompted us to investigate if the contemporary *pax5* and *etv6* knock-down could cooperate to alter the expression of B cell markers. Hence, we injected *pax5* and *etv6* morpholino together at lower doses, but the contemporary *pax5* and *etv6* knock-down did not cause a greater alteration of B cell markers expression compared to the single morphant embryos. Finally, we injected *PAX5/ETV6* mRNA in zebrafish embryos and we verified that the expression of this aberrant gene is able to induce the same molecular alteration observed *in vitro*, suggesting that zebrafish could be a suitable model to study BCP-ALL related to *PAX5/ETV6* fusion gene.

Materials and methods

Zebrafish lines and maintenance

Zebrafish (*Danio rerio*) embryos were raised and maintained under standard conditions and national guidelines (Italian decree 4th March 2014, n.26). All experimental procedures were approved by IACUC (Institutional Animal Care and Use Committee). Zebrafish AB wild-type strains were obtained from the Wilson lab, University College London, London, United Kingdom. For in vivo observations and imaging, embryos beyond 24 hpf were washed, dechorionated and anaesthetized with 0.016% tricaine (ethyl 3-aminobenzoate methanesulfonate salt; Sigma-Aldrich) before observations and picture acquisition. Embryos were staged according to morphological criteria (Kimmel *et al.*, 1995).

Whole mount *in situ* hybridization, embryo sectioning and imaging

Whole mount *in situ* hybridizations were carried out as described (Thisse and Thisse, 2008). The riboprobes were synthesized using the Ambion® MAXIscript® SP6/T7 *in Vitro* Transcription Kit (Thermo Fisher Scientific) as described in the following papers: *pax5* (Pfeffer *et al.*, 1998), *lck* (Zhang *et al.*, 2013). Images of stained embryos were taken on a Leica MZFLIII epifluorescence stereomicroscope equipped with a DFC 480 digital camera and LAS Leica imaging software (Leica, Wetzlar, Germany). Some of the hybridized embryos were then embedded in paraffin (Paraplast plus, Bio Optica) and sectioned (8μm) on a microtome (Leitz 1516). The slides were mounted with Eukitt (Bio Optica) and all sections were observed using a Leica DM6000B microscope equipped with a DFC 480 digital camera and LAS Leica imaging software (Leica, Wetzlar, Germany).

Real-time quantitative PCR assay and semi-quantitative RT-PCR

Total RNA was isolated from embryos at different developmental stages using the SV Total RNA isolation System kit (Promega, Madison, Wisconsin, USA). After treatment with DNase I RNase-free (Roche, Basel, Switzerland), 1 μg of RNA was reverse-transcribed using the ImProm-II™ Reverse Transcription System (Promega) according to manufacturer's instructions. Real-time analysis was performed on Light Cycler 480II with Universal Probe Master system (Roche Diagnostics; F. Hoffmann-La Roche Ltd.). Primers and probes were selected according to the Software Probe Finder (Roche Diagnostics) and are reported in Supplementary Table S1. Data were expressed using the comparative ΔΔCt method, using *rpl8* gene as reference and *ctrl*-MO or *GFP* mRNA injected

embryos as standardization control; both t test and SD values refer to triplicates of a single experiment and N=3 biological independent experiments were performed for each gene.
To assess the non-specific activation of p53, semi-quantitative RT-PCR were performed with cDNA obtained from *etv6* morphants at 24hpf. *p53* and *Δ113-p53* were subjected to 20 or 25 amplification cycles using primers reported in (Robu *et al.*, 2007).

Morpholino microinjection

Antisense morpholinos were purchased from Gene Tools (LLC, Philomath, OR). Morpholinos were diluted in Danieau solution (Nasevicius and Ekker, 2000) and injected into 1-to-2 cell-stage embryos using Eppendorf FemtoJet Micromanipulator 5171. Rhodamine dextran (Molecular Probes, Life technology) was co-injected as dye tracer. *etv6* knockdown was performed injecting 0,5pmol/embryo of a translation blocking MO (*etv6*-MO 5'-CATGTCTCGTTGAAATTCAGGAAGT-3') previously described (Roukens *et al.*, 2010). *pax5* knockdown was performed injecting 0,5pmol/embryo of a translation blocking MO (*pax5*-MO 5'-CGTGTTTGCAGTGGATTTCCATCGT-3'). A standard control oligo (ctrl-MO, 5'-CCTCTTACCTCAGTTACAATTTATA-3', against human *β-globin* gene) with no target in zebrafish embryos, was also used, to check for non-specific effects due to the injection procedure. Co-injection of *etv6*-MO with 0,75 pmol/embryo p53-MO was performed to abolish aspecific effects (Robu et al., 2007).

Western blot analysis

Embryo extracts were prepared as previously described (Bellipanni *et al.*, 2000). Western blots were performed as previously described (Fazio *et al.*,2008). Primary antibody: Anti-beta-actin (AC-15, Sigma-Aldrich), anti-N-term PAX5 (AB4227, Chemicon), anti-LCK (Cell Signaling Technologies), anti-LCK^{Y394} (Cell Signaling Technologies) anti-STAT5 (Cell Signaling Technologies), anti-STAT5^{Y694} (Merck-Millipore). Densitometry analyses were performed using ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA).

RNA microinjection

PAX5/ETV6 coding sequence was subcloned from MIGR1 vector (Fazio *et al.*, 2008) to pCS2+ plasmid and used as template for generating sense mRNA, *in vitro* synthesized using the mMessage mMachine kit (Ambion) according to the manufacturer's instructions. 200 pg/embryo or 250 pg/embryo of *PAX5/ETV6* mRNA, diluted in RNase-free water, were injected into one-or two-cell-

stage embryos with rhodamine dextran as dye tracer (Molecular Probes, Life technology). The *GFP* mRNA was injected as negative control.

Results

pax5 is expressed in the pancreas of zebrafish larvae

In order to verify that *pax5* is expressed in organs involved in hematopoiesis during zebrafish development, we performed whole mount *in situ* hybridization (WISH) from 24 hpf (hours post fertilization) to 5 dpf. As it has been previously reported, *pax5* is expressed in the midbrain-hindbrain boundary from the 5 somite stage onwards. Later, *pax5* expression is observed in the anterior part of the otic vesicle from the 17 somite stage to 72 hpf (Pfeffer *et al.,* 1998; Kwak *et al.,* 2006; data not shown). Starting from about 60 hpf, we observed a weak staining of the *pax5* probe in the gut and hybridization signals in the liver and in the pancreas (Fig. 1A-B), which has been proposed as a transient site of B cell development in zebrafish (Danilova and Steiner, 2002), in addition to the previously reported tissues. This expression pattern is maintained at least until 5 dpf (Fig. 1C-D).

The B cell markers *igh*μ, *cd79a, cd79b, blnk, ebf1a* and *ebf1b* are conserved in zebrafish and are highly expressed in larval developmental stages

Previously to this work, the only B cell specific marker known to be conserved in zebrafish was *igh*μ (Danilova and Steiner, 2002). Hence, to gain new insights into B cell development and to identify the better stage to perform loss of function experiments we characterized the expression of several B cell markers by qRT-PCR. Specifically, besides *igh*μ (fig. 2B), the analysed markers are *cd79a* (fig. 2C), *cd79b* (fig. 2D), *blnk* (fig. 2A) and *ebf1*, which in zebrafish is duplicated in *ebf1a* (fig. 2E) and *ebf1b* (fig. 2F). The human *CD79a* and *CD79b* genes encode respectively Igα and Igβ that, together with IgH protein, are components of the BCR, *BLNK* encodes an adaptor protein involved in B cell receptor signaling whereas EBF1 is a transcription factor necessary for B cell specification. To note, the expression of *BLNK*, *CD79a* and *EBF1* is known to be directly activated by PAX5. qRT-PCR assays showed that the most of these genes are highly expressed in late larval stages, starting from about 72 hpf, during zebrafish development (fig. 2). Thus, as a compromise between the morpholino efficiency, which is most effective until about 72 hpf (Nasevicius and Ekker, 2000), and the increase of B cell markers expression, we decided to perform functional experiments at 5 dpf.

pax5 knockdown alters the expression of B cell markers

Since it is not known if the role of *pax5* in B cell development is conserved in zebrafish, we analyzed if *pax5* knockdown causes the alteration of B cell markers expression.

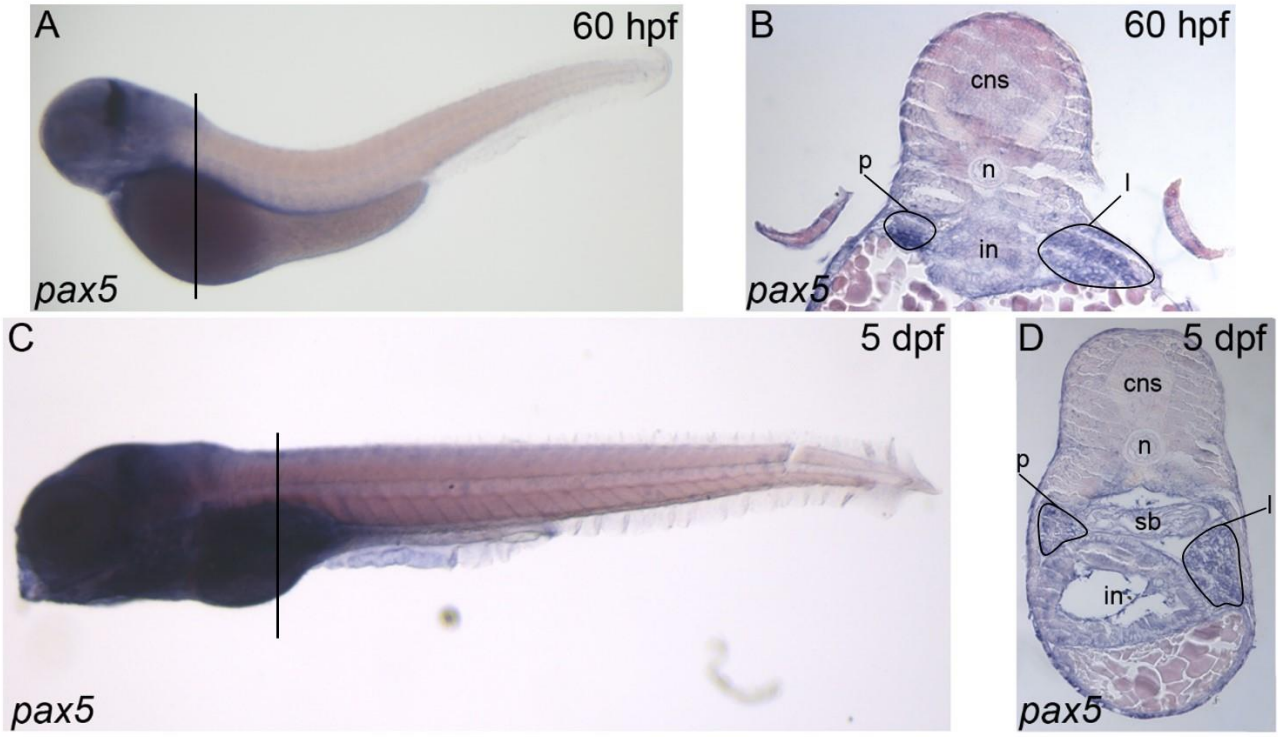

Figure 1: *pax5* is expressed in the pancreas of zebrafish larvae. WISH performed on zebrafish embryos at 60 hpf and 5 dpf. (B, D) Transversal sections showing *etv6* expression in the liver (l), in the pancreas (p) and in the intestine (in) at 60 hpf (B) and 5 dpf (D). (A, C) Lateral views; dorsal is up, anterior is left. (B, D) Transversal section of the trunk; dorsal is up. Abbreviations: cns, central nervous system; in, intestine; l, liver; n, notochord; p, pancreas; sb, swim bladder.

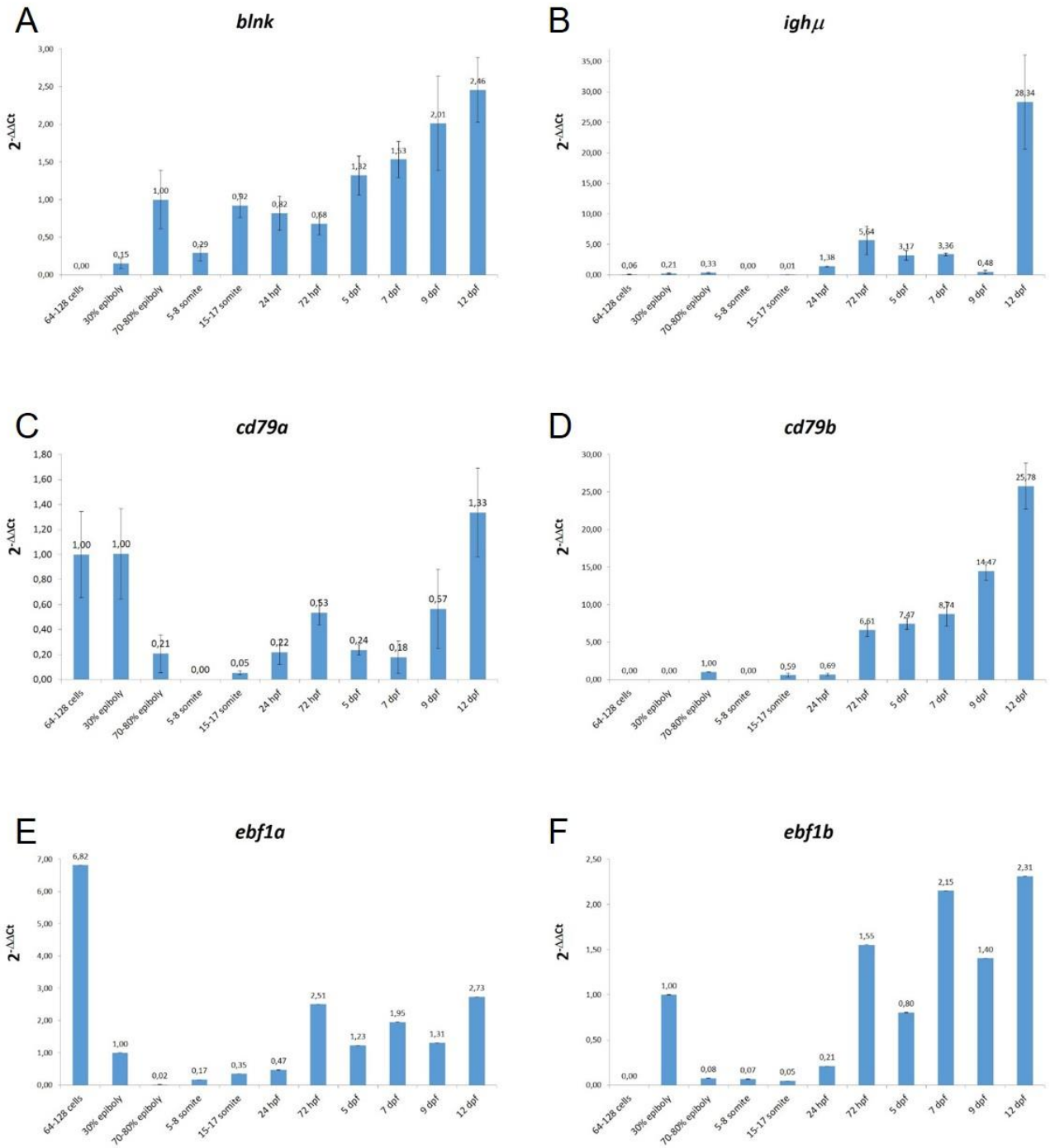

Figure 2: Analysis of B cell markers temporal expression. qRT-PCR assays of *blnk* (A), *ighμ* (B), *cd79a* (C), *cd79b* (D), *ebf1a* (E)and *ebf1b* (F) performed at the indicated developmental stages from 64-128 cells to 12 dpf.

pax5 loss of function experiments were performed by the injection of 0,5pmol/embryo of a morpholino designed to block *pax5* translation (*pax5*-MO). qRT-PCR assays showed that *pax5* morpholino injection caused the downregulation of *igh*μ (FC = 0,2 $p < 0,01$; Fig. 3C) and *cd79a* (FC = 0,3 $p < 0,01$; Fig: 3A), as well as the upregulation of *cd79b* (FC = 2,16 $p < 0,05$; Fig. 3B) compared to control embryos. However, there was no significant alteration of the expression of *blnk* (Fig. 3D), *ebf1a* and *ebf1b* (Fig. 3E and 3F) in *pax5* morphants compared to control embryos.
These findings suggest that the role of *pax5* in B cell development could be, at least in part, conserved in zebrafish.

etv6 knockdown causes the deregulation of the expression of B cell markers

In mammals *Etv6* is required for establishing hematopoiesis within the bone marrow but is dispensable for primitive and definitive embryonic hematopoiesis that occur in yolk sac and fetal liver (Wang *et al.*, 1997; Wang *et al.*, 1998; Hock *et al.*, 2004). Recently, it has been reported that in zebrafish *etv6* is involved in both primitive and definitive hematopoiesis. Moreover, *etv6* knockdown caused the alteration of lymphoid precursors markers *rag1* and *ikaros* (Rasighaemi *et al.*, 2015). Hence, we tested if *etv6* loss of function could induce the deregulation of B cell markers. To perform *etv6* knockdown, we injected 0,5 pmol/embryo of a translation blocking morpholino that it has been already published and validated (Roukens *et al.*, 2010). However, the injection of *etv6* morpholino (*etv6*-MO) caused morphological alterations that were not previously described. Specifically, *etv6* knockdown caused apoptosis in brain tissue and curved tail at 24 hpf (Fig. S1A). It is known that some morpholino molecules could elicit p53 off-target activation causing nonspecific morphological defects similar to those observed in *etv6* morphants (Robu *et al.*, 2007). We verified that *etv6*-MO causes the upregulation of an N-terminal truncated p53 isoform that is diagnostic for the nonspecific p53 activation (Fig, S1B; Robu *et al.*, 2007); moreover, *etv6* morphants phenotype is rescued by *p53*-MO coinjection (Fig. S1A). Consequently, we decided to coinject *etv6*-MO and *p53*-MO in the subsequent experiments to suppress off-target effects and facilitate the study of specific loss of function phenotypes.
Using qRT-PCR analysis we observed the deregulation of several B cell markers in *etv6* morphants. Indeed, in *etv6*-MO injected embryos *igh*μ (FC = 0,28 $p < 0,05$; Fig. 4C), *cd79a* (FC = 0,31 $p < 0,05$; Fig. 4A) and *blnk* (FC = 0,62 $p < 0,05$; Fig. 4D) were downregulated, whereas *cd79b* (FC = 1,27 $p < 0,01$; Fig. 4B) was slightly upregulated. However, the expression of *ebf1a* and *ebf1b* was comparable between control and *etv6* morphant embryos (Fig. 4E and 4F). Thus, our results indicate that *etv6* is involved in the regulation of the expression of *igh*μ , *cd79a*, *blnk* and *cd79b*.

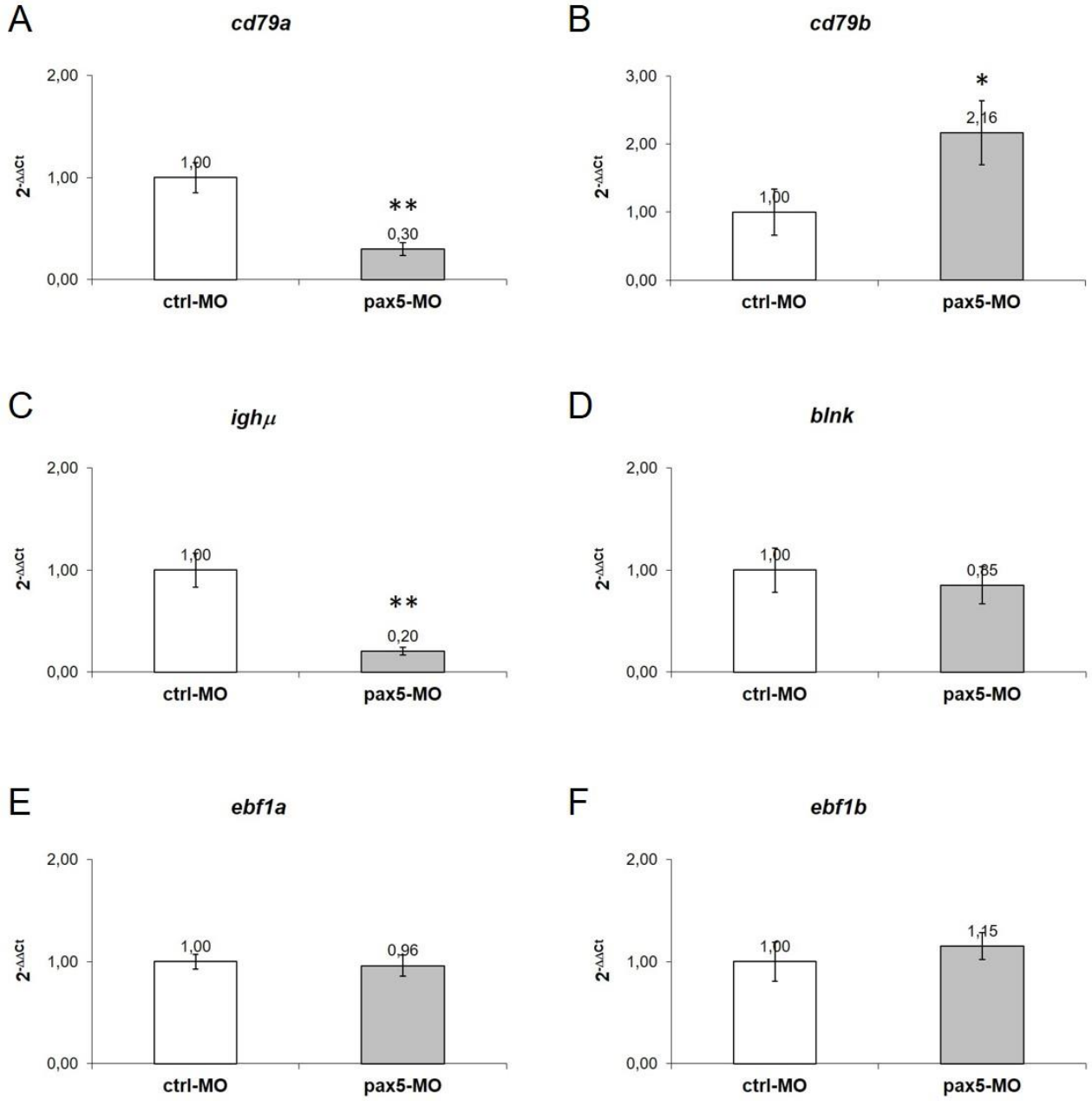

Figure 3: *pax5* knockdown causes the alteration of B cell markers expression. qRT-PCR analysis showing the downregulation of *cd79a* (A) and *ighμ* (C) as well as the upregulation of *cd79b* (B) in *pax5*-MO injected embryos at 5 dpf. The expression of *blnk* (D), *ebf1a* (E) and *ebf1b* (F) is comparable between control and *pax5* morphant embryos at 5 dpf. *t* test: * = $p < 0,05$; ** = $p < 0,01$.

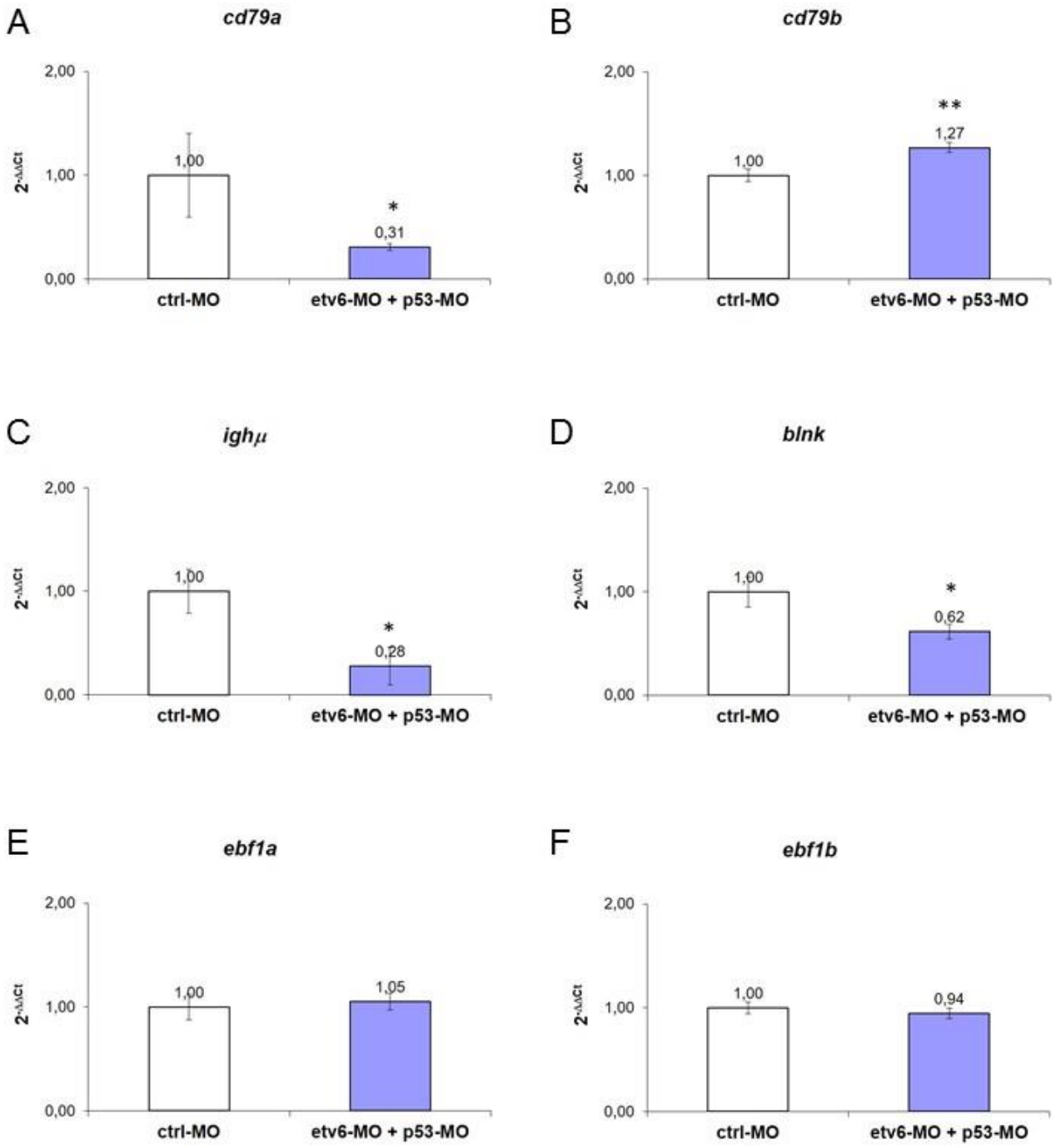

Figure 4: *etv6* loss of function determines the deregulation of B cell markers expression. qRT-PCR analysis showing the downregulation of *cd79a* (A), *ighμ* (C) and *blnk* (D) as well as the upregulation of *cd79b* (B) in *etv6* morphants at 5 dpf. The expression of *ebf1a* (E) and *ebf1b* (F) is comparable between control and *pax5* morphant embryos at 5 dpf. *t* test: * = $p < 0,05$; ** = $p < 0,01$.

pax5 and _etv6_ do not cooperate in the regulation of _igh_μ and _cd79a_ expression

It has been proposed that the loss of one wild type *PAX5* allele and downregulation of *PAX5* expression or reduced levels of PAX5 protein in patients carrying either a mutation, a deletion or a fusion gene could be a mechanism of leukemogenesis (Mullighan *et al.,* 2007; An *et al.,* 2008). However, additional genetic events further reducing PAX5 activity or other genetic lesions are required to establish the leukemic clone (Cobaleda *et al.,* 2007; Mullighan *et al.,* 2007; Shah *et al.,* 2013; Auer *et al.,* 2014). We observed that *etv6* knockdown caused the alteration of the expression of some B cell markers which is impaired also in *pax5* morphants and we wondered if the contemporary mono-allelic losses of *PAX5* and *ETV6* in BCP-ALL patients could influence the expression of these genes. We consequently decided to investigate if the contemporary *pax5* and *etv6* knock-down could cooperate to alter the expression of *igh*μ and *cd79a*, which are both downregulated in *pax5* and *etv6* single morphants. The ideal setting to perform this experiment is to coinject subphenotypical doses of both morpholinos. Thus, we coinjected 0,2 pmol/embryo of both *pax5* and *etv6* morpholinos because the expression of *igh*μ in both *pax5* and *etv6* single morphants as well as *cd79a* in *etv6* single morphants is still slightly downregulated but these variations are nevertheless not statistically significant when compared with control embryos. qRT-PCR assays showed that the coinjection of *pax5* and *etv6* morpholinos did not cause significant alteration of *igh*μ (Fig.S2B) and *cd79a* (Fig.S2A) expression if compared with single morphants or control embryos. Hence, these experiments seem to exclude a possible cooperation between *pax5* and *etv6* in the regulation of *igh*μ and *cd79a* expression.

Transient expression of PAX5/ETV6 in zebrafish embryos

Since we aimed to verify if zebrafish could be a suitable model to study the role of *PAX5/ETV6* fusion gene in BCP-ALL, we transiently expressed *PAX5/ETV6* in zebrafish embryos by the injection of its mRNA. To synthesize the mRNA, we previously sub-cloned the human *PAX5/ETV6* coding sequence from the MIGR vector (Fazio *et al.,* 2008) into the pCS2+ plasmid. The transient expression of *PAX5/ETV6* was obtained by the injection of two doses of the mRNA, which was 200 pg/embryo and 250 pg/embryo, while we injected the mRNA encoding the GFP protein as negative control. The expression of PAX5/ETV6 protein was confirmed by western blot analysis of *PAX5/ETV6* mRNA injected embryo (Fig.S3A). We verified that *PAX5/ETV6* is correctly transcribed and translated *in vivo* also by injection of pCS2+ vector containing the *PAX5/ETV6* coding sequence (Fig.S3B), which induced a strong expression of the fusion protein due to its CMV promoter. Embryos injected with

either 200 or 250 pg/embryo of *PAX5/ETV6* mRNA did not show morphological alterations until 5 dpf, at this stage we observed a mild defect in yolk absorption (data not shown).

PAX5/ETV6 did not cause the alteration of B cell markers in zebrafish embryos

The expression of PAX5/ETV6 in murine pre-BI cells causes the downregulation of *Cd79a* and *Blnk* determining the block of pre-BCR assembly (Fazio *et al.,* 2008). Thus, we analyzed their expression in *PAX5/ETV6* mRNA injected embryos by qRT-PCR analysis at 5 dpf, however, we did not observe a statistically significant alteration of *cd79a* and *blnk* expression levels compared to control embryos (Fig.S4A, C). Moreover, also the expression of both *ighμ* and *cd79b* was comparable between control and *PAX5/ETV6* mRNA injected embryos (Fig.S4B, D).

The injection of *PAX5/ETV6* mRNA induced the upregulation of *lck* and the activation of lck protein

PAX5/ETV6 caused the upregulation of *Lck* mRNA and enhanced Lck kinase activity *in vitro* and in BCP-ALL patients with *PAX5* fusion genes (Cazzaniga *et al.,* 2015). Thus, we analyzed the expression of *lck* by WISH assays at 5 dpf in *PAX5/ETV6* mRNA injected embryos. We found that *lck* expression in the thymus is upregulated and expanded in the 21% (n=19; Fig. 5B) and in the 38% (n=13; Fig. 5C) of embryos injected with 200 pg/embryo and 250 pg/embryo respectively of *PAX5-ETV6* mRNA compared to control embryos.

Lck kinase activity is regulated through different phosphorylation events and the autophosphorylation of the tyrosine Y394 in the activation loop enhances the kinase activity (Hui and Vale, 2014). This tyrosine phosphorylation site is conserved in zebrafish, however it is located in the position 387 in the predicted lck protein (data not shown). To verify if PAX5/ETV6 causes the activation of Lck protein also in zebrafish, we performed western blot assays with protein extracts of *PAX5/ETV6* mRNA injected embryos using an anti-Lck^{Y394} antibody. The level of Lck phosphorylated protein, expressed as the ratio between Lck^{Y394} and Lck total protein, is increased of the 18% in embryos injected with 200 pg/embryo of *PAX5-ETV6* mRNA compared to control embryos while the injection of 250 pg/embryo of *PAX5/ETV6* mRNA determined the 54% increase of Lck phosphorylated protein (Fig. 5D).

These results suggest that *PAX5/ETV6* expression in zebrafish embryos causes the upregulation of *lck* expression and the activation of Lck protein in a dose dependent manner.

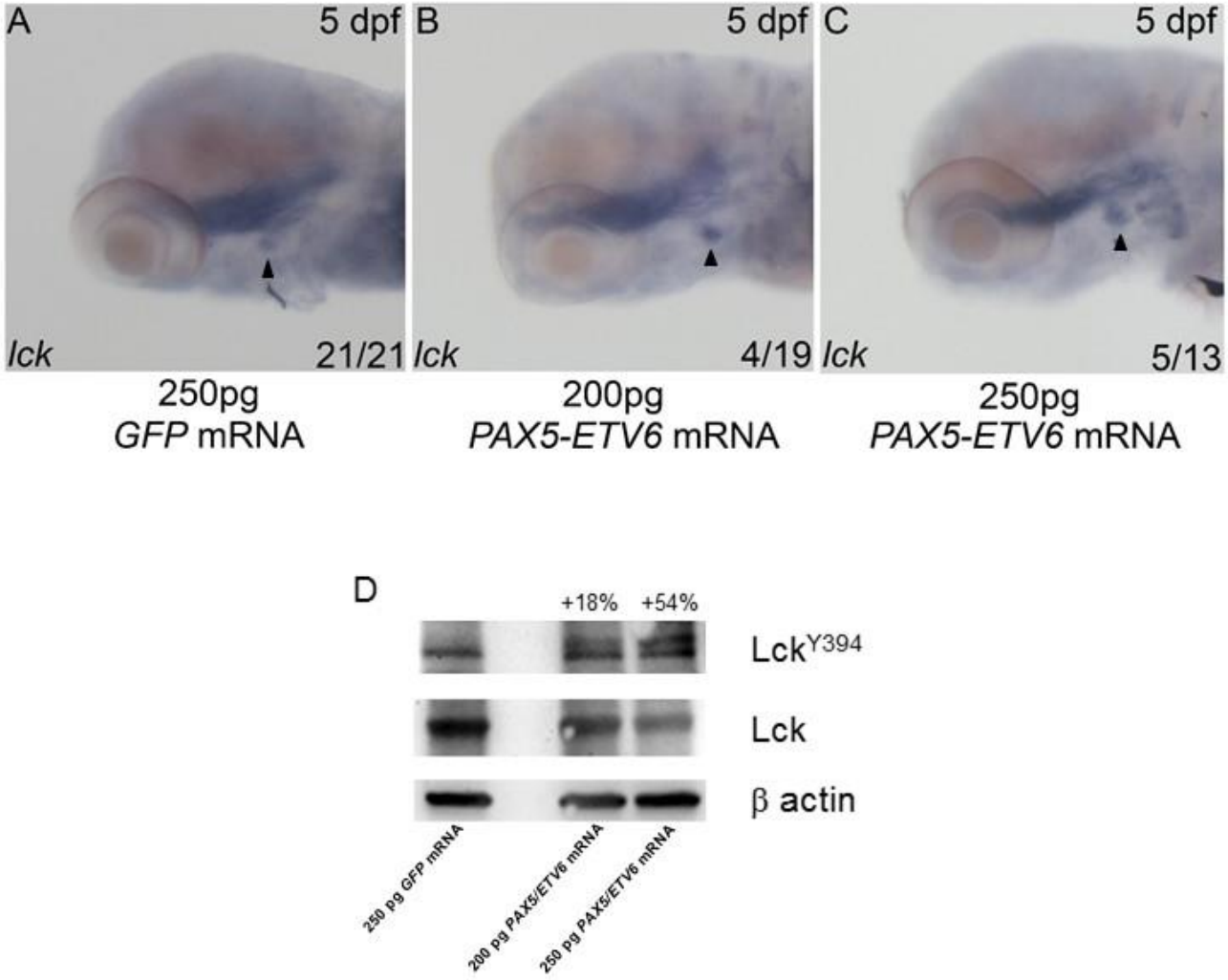

Figure 5: PAX6/ETV6 causes the upregulation of *lck* mRNA and the phosphorylation of Lck protein. (A-C) WISH assays showing the expression of *lck* in the thymus, indicated by the arrowhead, at 5 dpf. *lck* expression is increased and expanded in embryos injected with 250 pg/embryo (C) or 200 pg/embryo (B) of *PAX5/ETV6* mRNA compared to controls injected with 250 pg/embryo of *GFP* mRNA. (A-C) Lateral views of the head, dorsal is up, anterior is left. (D) Western blot assays showing the expression of phosphorylated Lck^{Y394}, total Lck and βactin as internal control at 5 dpf. Lck^{Y394} phosphorylated protein is increased of 18% in embryos injected with 200 pg/embryo of *PAX5/ETV6* mRNA and of 54% in embryos injected with 250 pg/embryo of *PAX5/ETV6* mRNA.

PAX5/ETV6 caused the activation of stat5 protein and the upregulation of stat5 effectors

The activation of Lck protein induced by PAX5/ETV6 in murine pre-BI cells is responsible for the phosphorylation of the STAT5 tyrosine residue 694, leading to the activation of the protein and the upregulation of the STAT5 effectors *cMyc* and *Ccnd2* (Cazzaniga *et al.,* 2015). The mechanism of stat5 activation through the phosphorylation of Y694 is conserved in zebrafish (Lewis *et al.,* 2006). Therefore, we performed western blot assays at 5 dpf using an anti-STAT5^{Y694} to test if the injection of *PAX5-ETV6* mRNA is able to induce stat5 activation in zebrafish embryos. The injection of 200 pg/embryo of *PAX5-ETV6* mRNA did not cause an increase of STAT5^{Y694} phosphorylated protein. However, the level of STAT5^{Y694} was increased by 223% in embryos injected with 250 pg/embryo of *PAX5-ETV6* mRNA compared to control embryos (Fig. 6A).

The homologues of the murine STAT5 effectors *cMyc* and *Ccnd2* are duplicated in zebrafish and we analyzed the expression of *cmyca, cmycb* and *ccnd2a* genes by qRT-PCR assays at 5dpf in embryos injected with 200 and 250 pg/embryo of *PAX5-ETV6* mRNA. The expression of all the stat5 effectors analyzed was statistically significant upregulated at both doses of *PAX5-ETV6* mRNA showing a dose dependent effect (Fig. 6B-D).

Thus, PAX5/ETV6 expression in zebrafish embryos is able to induce the phosphorylation and activation of stat5, as well as the upregulation of the stat5 effectors *cmyca, cmycb* and *ccnd2a*.

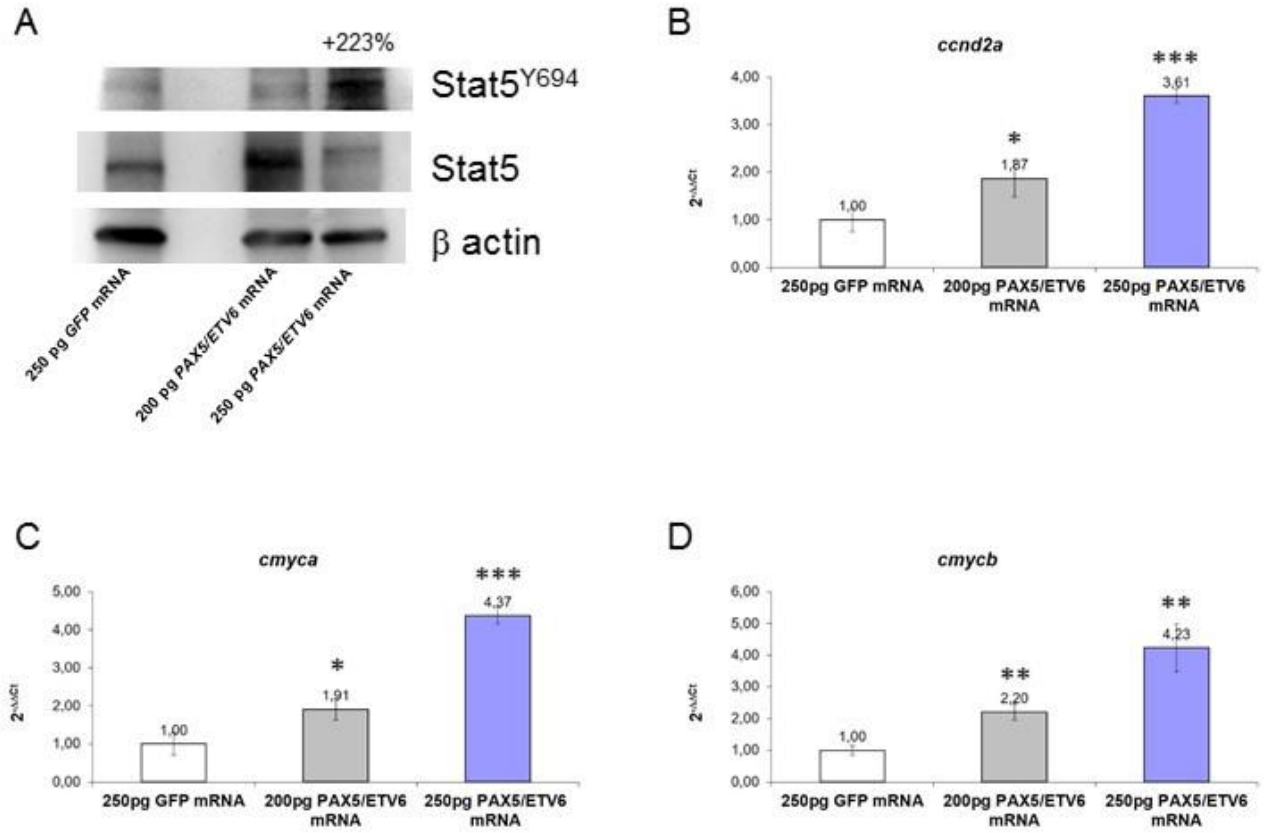

Figure 6: PAX6/ETV6 induces the phosphorylation of Stat5 protein and the upregulation of *ccnd2a*, *cmyca* and *cmycb*. (A) Western blot assays showing the expression of phosphorylated Stat5^{Y694}, total Stat5 and βactin as internal control. Stat5^{Y694} phosphorylated protein is increased of 323% in embryos injected with 250 pg/embryo of *PAX5/ETV6* mRNA. (B-D) qRT-PCR analysis showing the upregulation of *ccnd2a* (B), *cmyca* (C) and *cmycb* (D) in embryos injected with *PAX5/ETV6* mRNA at 5dpf. The level of *ccnd2a* (B), *cmyca* (C) and *cmycb* (D) increase with the dose of *PAX5/ETV6* mRNA injected. *t* test: * = $p < 0,05$; ** = $p < 0,01$; *** = $p < 0,001$.

Discussion

Zebrafish has proven to be a useful model to study physiological hematopoietic processes as well as pathological alterations involved in human hematological malignancies. The capability to reproduce human pathologies is due to the high conservation of the blood cell lineages and the molecular processes that regulate hematopoiesis (Lieschke and Currie, 2007; Paik and Zon, 2010; Jing and Zon, 2011). Many efforts have been made to deepen the knowledge of zebrafish hematopoiesis, however very little is known about zebrafish B cell development. Thus, we searched the zebrafish homologues of several mammal B cell markers and we found that *cd79a, cd79b*, *blnk* and *ebf1*, which is duplicated in *ebf1a* and *ebf1b*, are conserved in zebrafish. The expression analysis of these genes, together with the previously characterized *ighμ* (Danilova and Steiner, 2002), showed that the majority of them are highly expressed during larval stages, which is consistent with previous findings that put the onset of B cell development at 4 dpf and the presence of clearly identifiable *ighμ* expressing cells at about 10-19 dpf (Danilova and Steiner, 2002; Zapata *et al.*, 2006). Moreover, WISH analysis showed that *pax5* is expressed in the pancreas of zebrafish larvae, supporting the hypothesis proposed by Danilova and Steiner (2002) that the pancreas could be a transient site of B cell development in this teleost fish (Danilova and Steiner, 2002).

In mammals *PAX5* is essential for B cell commitment and for the maintenance of B cell identity throughout B cell maturation (Cobaleda *et al.*, 2007). However, there are no published data suggesting that the role of *pax5* in B cell is conserved in zebrafish. Thus, before testing if zebrafish could be a good model system to reproduce *PAX5/ETV6* related leukemia, it has been necessary to verify if *pax5* is involved in zebrafish B cell development. *pax5* knockdown by the injection of a translation blocking morpholino caused the alteration of several component of the B cell receptor, as assessed by qRT-PCR assays. Specifically, *pax5* loss of function determined the downregulation of *ighμ* and *cd79a*, as well as the upregulation of *cd79b*. Interestingly, in mammals *CD79a* is a direct target of *PAX5* (Nutt *et al.*, 1998). However, the expression of *blnk*, *ebf1a* and *ebf1b*, which are directly activated by *PAX5* in mammals (Schebesta *et al.*, 2002), was not altered by *pax5* knockdown. Therefore, it is possible that in zebrafish *pax5* is not involved in the activation of *blnk, ebf1a* and *ebf1b*. However, it is also likely that the residual pax5 protein still present in *pax5* knockdown embryos is sufficient to induce a normal expression of *blnk, ebf1a* and *ebf1b*. Overall, our genetic evidences suggest that *pax5* role in B cell development could be, at least in part, conserved in zebrafish. Since B cell emergence is a late event during zebrafish development, it has been necessary

to establish *pax5* mutant lines in order to study B cell phenotype in larval stages and adult fish to better characterize the role of *pax5* in zebrafish B cell development.

Patients carrying either a mutation, a deletion or a fusion gene involving *PAX5* are characterized by the significant downregulation of *PAX5* expression or reduced levels of PAX5 protein and corresponding activity (Mullighan *et al.,* 2007; An *et al.,* 2008). This evidences, in addition to the finding that some PAX5 fusion proteins act in a dominant negative manner, suggest that *PAX5* haploinsufficiency could be a mechanism of leukemogenesis. However, B cell development occur normally in heterozygous *Pax5*$^{+/-}$ mice and human individuals carrying the heterozygous inherited *PAX5* mutation 547G>A are mostly phenotypically normal in B cell development and all family members who develop BCP-ALL have also the concomitant deletion of the wild-type *PAX5* allele (Urbanek *et al.,* 1994; Shah *et al.,* 2013; Auer *et al.,* 2014). Hence, additional genetic events further reducing PAX5 activity or other genetic lesions are required to establish the leukemic clone (Cobaleda *et al.,* 2007; Mullighan *et al.,* 2007; Shah *et al.,* 2013; Auer *et al.,* 2014). BCP-ALL patients carrying *PAX5/ETV6* fusion are also characterized by mono-allelic losses of *PAX5* and *ETV6* genes. Therefore, the concomitant deletion of one wild type of both these genes could have a role in the pathogenesis of leukemia. Since we found that *etv6* knockdown caused the alteration of the expression of some B cell markers which are impaired also by *pax5* loss of function, we investigated if the contemporary *pax5* and *etv6* knock-down could cooperate to alter the expression of these genes. However, the coinjection of lower doses of *pax5* and *etv6* morpholinos did not cause significant alteration of *igh*μ and *cd79a*, thus excluding a possible cooperation between *pax5* and *etv6* in the regulation of *igh*μ and *cd79a* expression.

The final aim of this study was to test if the expression of the human *PAX5/ETV6* fusion gene in zebrafish embryos is able to induce the same molecular alterations observed *in vitro* in murine pre-BI cells (Fazio *et al.,* 2008; Fazio *et al.,*2013; Cazzaniga *et al.,* 2015). One of the alterations caused by PAX5/ETV6 in pre-BI cells is the block of pre-BCR assembly through the downregulation of the PAX target genes *Cd79a* and *Blnk* (Fazio *et al.,* 2008). However, the expression of these genes, as well as *igh*μ and *cd79b*, is not deregulated by the expression of PAX5/ETV6 in zebrafish embryos. Now it is thought that leukemia is a multi-step process in which multiple genetic lesions cooperate to oncogenic transformation, in fact many of the known alterations alone are not sufficient to induce leukemia in experimental models (Greaves *et al*., 2003; Mullighan *et al*., 2007; Pui *et al.,* 2008; Inaba *et al.,* 2013). Thus, it is possible that in zebrafish the expression of *PAX5/ETV6* fusion gene alone is not sufficient to cause the alteration of the analysed B cell markers *in vivo* or a longer experimental time is needed for leukemia development.

PAX5/ETV6 expression confers survival advantages to pre-BI cells due to the activation of Lck protein, that in turn induces the phosphorylation and activation of STAT5 leading to the overexpression of STAT5 effectors *cMyc* and *Ccnd2* (Cazzaniga *et al.*, 2015). We thus tested if the expression if the injection of *PAX5/ETV6* mRNA is able to cause the same alterations in zebrafish embryo and we observed that *lck* expression is upregulated in a dose dependent manner. Moreover, the amount of Lck protein phosphorylated in the activator residue Y394 is increased in *PAX5/ETV6* mRNA injected embryos in a dose dependent manner, therefore indicating that PAX5/ETV6 can induce the activation of Lck protein in zebrafish.

Importantly, the zebrafish homologues of the murine STAT5 effectors *cMyc* and *Ccnd2* are upregulated in a dose dependent manner in embryos injected with *PAX5/ETV6* mRNA, suggesting that PAX5/ETV6 expression activates the same pathways involved in the acquisition of the survival advantages showed by murine pre-BI cells. However, the level of Stat5 activated protein is increased only in embryos injected with the higher dose of the *PAX5/ETV6* mRNA. It is conceivable that at the lower dose of *PAX5/ETV6* mRNA the upregulation of *cmyca*, *cmycb* and *ccnd2a* could be induced by the activation of other not yet identified pathways.

Overall, our results indicate that the molecular alterations caused by *PAX5/ETV6 in vitro* can be recapitulated in zebrafish, suggesting that zebrafish could be a good model system to study *in vivo* BCP-ALL associated to *PAX5/ETV6*. It will be useful to establish an inducible transgenic line to stably express the fusion gene also in later developmental stages and in adult fish to verify if longer expression of *PAX5/ETV6* can induce the development of overt leukemia. Such studies of the leukemic process could be useful for the identification of new potential therapeutic targets. Moreover, zebrafish embryos are permeable to soluble molecules dissolved in the culture medium and are particularly suitable for large scale drug screenings (Lieschke and Currie, 2007; Paik and Zon, 2010; Jing and Zon, 2011). Thus, zebrafish model of *PAX5/ETV6*-related BCP-ALL could be used to perform drug screenings *in vivo* in order to identify novel therapeutic compounds.

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Supplementary figure legends

Figure S1: *etv6*-MO induces the non-specific activation of *Δ113-p53*. (A) The injection of 0,5 pmol/embryo of *etv6*-MO causes apoptosis in brain tissue and curved tail at 24 hpf. These alterations are rescued by the co-injection of 0,75 pmol/embryo of *p53*-MO along with *etv6*-MO. (B) Semi-quantitative RT-PCR performed at 24 hpf showing that *etv6*-MO injection induces the upregulation of *Δ113-p53*, encoding a truncated p53 isoform which is considered diagnostic for non-specific p53 activation caused by some morpholino molecules (Robu *et al.*, 2007). Also the semi-quantitative RT-PCRs of *p53* and *βactin* are reported. *βactin* amplification at 25 cycles is shown to compare the saturation level.

Figure S2: *pax5* and *etv6* double knockdown does not cooperate to alter the expression of *ighμ* and *cd79a*. The coinjection of 0,2 pmol/embryo of *etv6*-MO with 0,2 pmol/embryo of *pax5*-MO did not induce the alteration of *ighμ* and *cd79a* expression at 5 dpf, as assessed by qRT-PCR assays.

Figure S3: Expression of PAX5/ETV6 in zebrafish embryos. Western blot assays performed with an anti-human-PAX5 antibody (Chemicon) showing the expression of PAX5/ETV6 protein in zebrafish embryos injected with 200 pg of *PAX5/ETV6* mRNA (A) and 100 pg of pCS2+ vector containing the *PAX5/ETV6* coding sequence. The protein extracts were obtained from embryos at 30 hpf (A) and 4 dpf (B).

Figure S4: The expression of B cell markers is not altered in embryos injected with *PAX5/ETV6* mRNA. The expression of *cd79a* (A), *cd79b* (B), *blnk* (C) and *ighμ* (D) at 5 dpf is comparable between embryos injected with *PAX5/ETV6* mRNA and *GFP* mRNA.

Supplementary figures

Figure S1

A

B

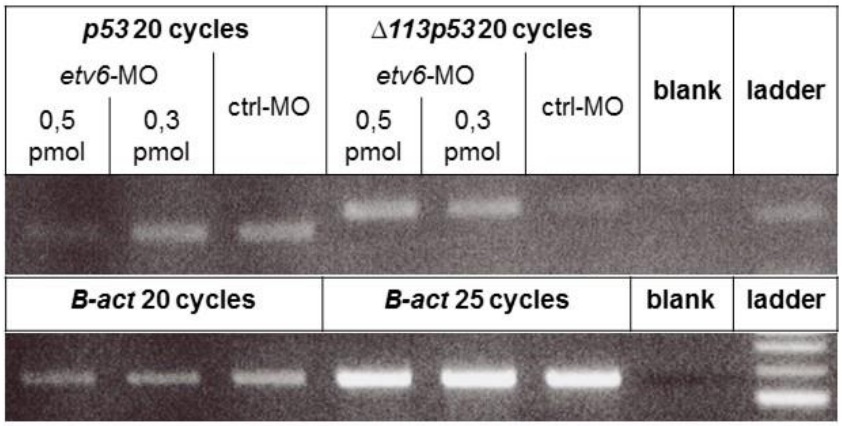
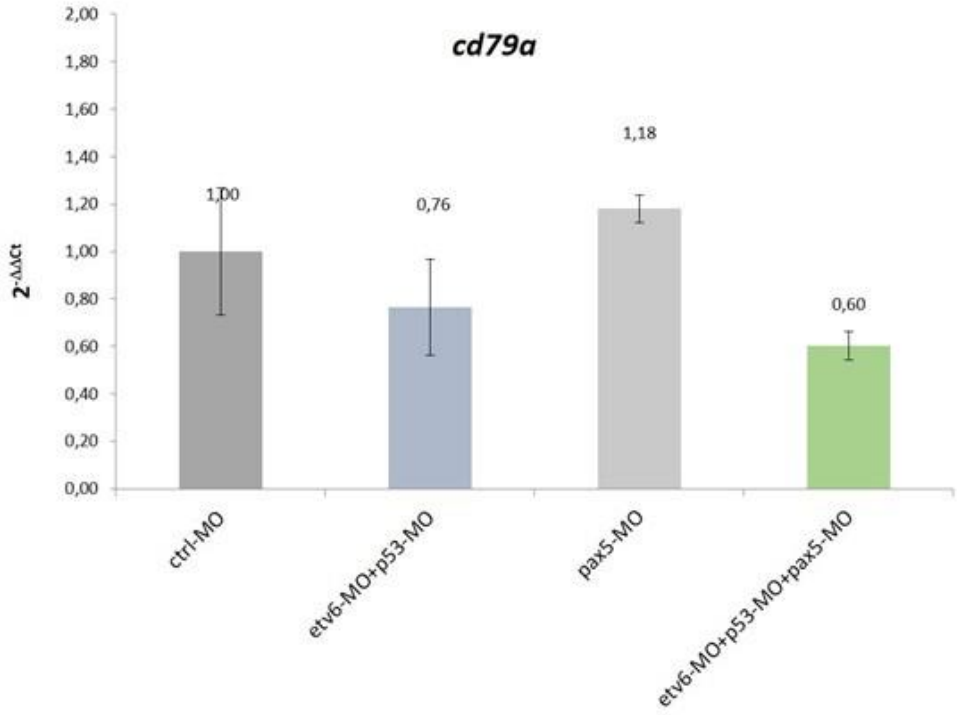


Figure S2

A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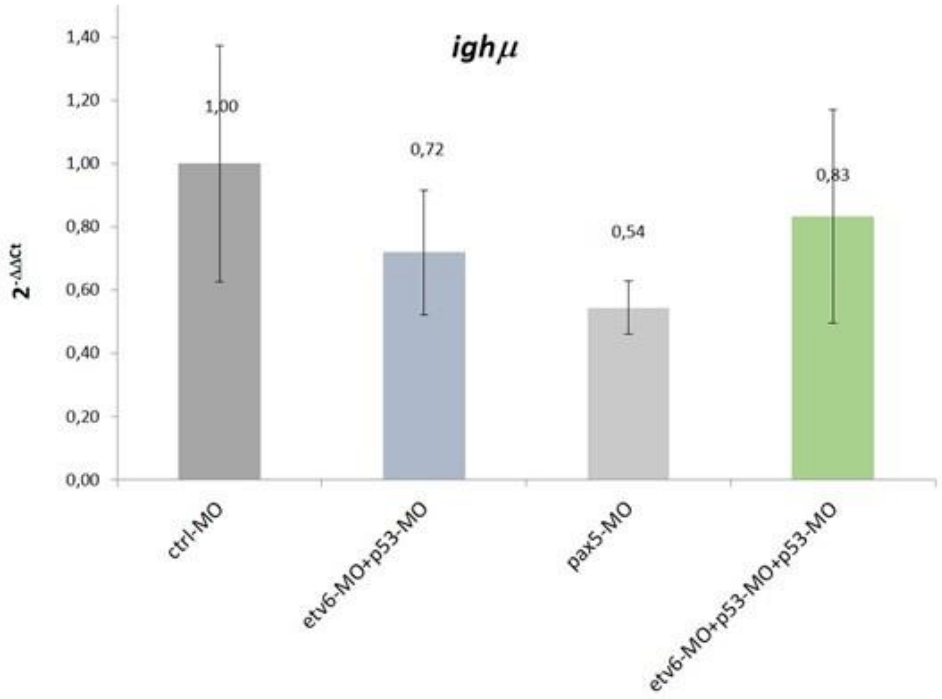
0,00
1,00
0,76
1,18
0,60
ctrl-MO
etv6-MO+p53-MO
pax5-MO
etv6-MO+p53-MO+pax5-MO

B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1,00
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ctrl-MO
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pax5-MO
etv6-MO+p53-MO+p53-MO

Figure S3

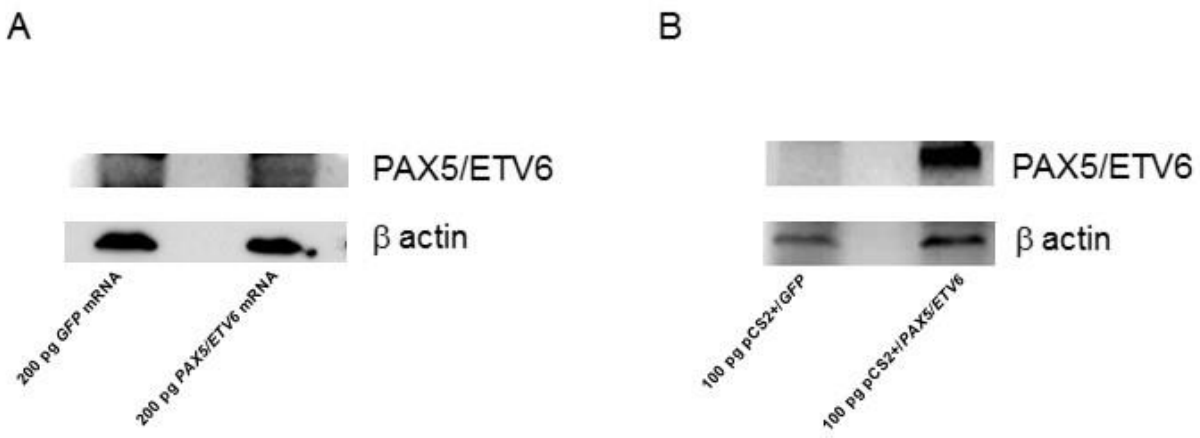
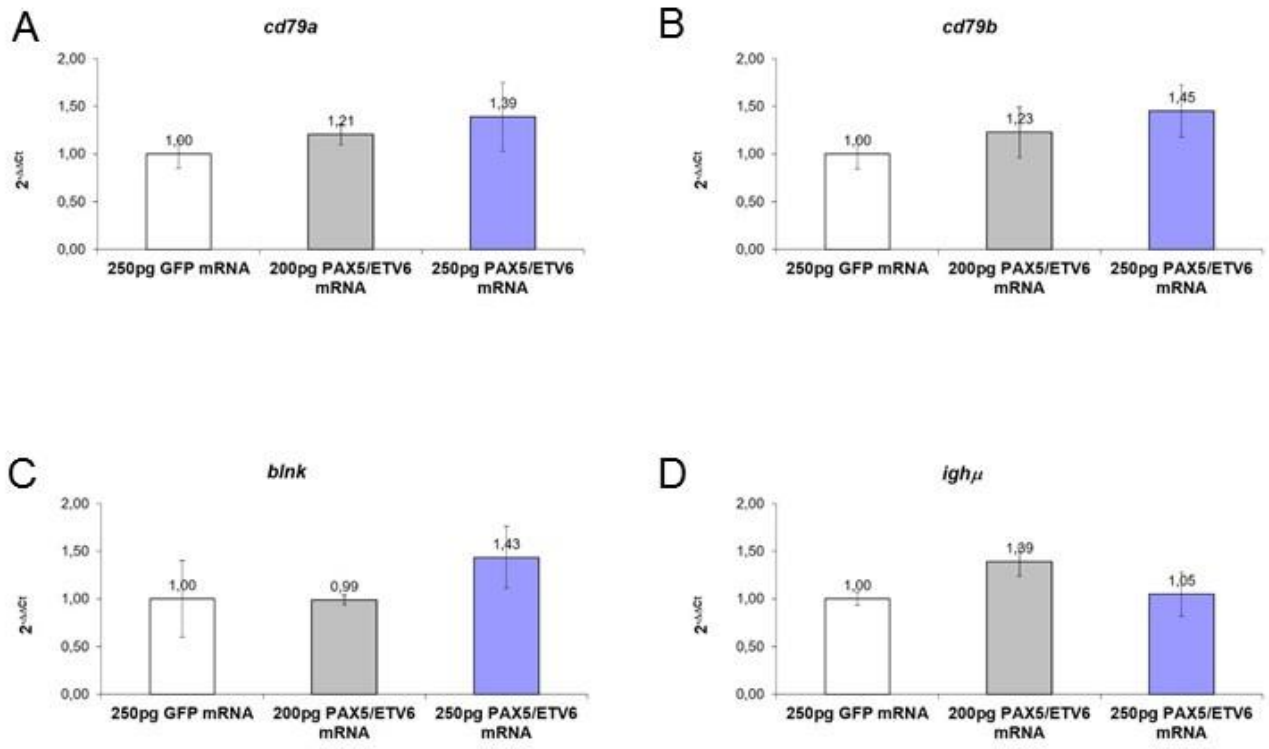

A
PAX5/ETV6
β actin
200 pg GFP mRNA
200 pg PAX5/ETV6 mRNA
B
PAX5/ETV6
β actin
100 pg pCS2+/GFP
100 pg pCS2+/PAX5/ETV6

Figure S4


A
cd79a
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B
cd79b
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C
blnk
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250pg PAX5/ETV6 mRNA
D
ighμ
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1,39
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200pg PAX5/ETV6 mRNA
250pg PAX5/ETV6 mRNA

Supplementary Table S1. List of primers and UPL probe number used.

Gene name	**Primer sequence**	**Probe number**
z_rpl8 fw	tcaagggaatcgtgaaggac	82
z_rpl8 rev	gggtcacgaaacatcacctt	
z_blnk fw	caaaatgaacaagcggctaat	47
z_blnk rev	gcggctctttcttcttcagtc	
z_ccnd2a fw	atcagaccgcaagaactgct	24
z_ccnd2a rev	tgacagctgctaggttccac	
z_cd79a fw	tgaataaactggacaggaagacag	69
z_cd79a rev	tgcagtcatccagattaagacc	
z_cd79b fw	cctcttggttttgtgcattg	6
z_cd79b rev	tcagcctcttctttcttctcca	
z_cmyca fw	ggtcctggacactccaccta	59
z_cmyca rev	tcctcctcctcatcctcttct	
z_cmycb fw	agcagtagtgacagcgaatcc	35
z_cmycb rev	ccgtgaccacgtcaatttct	
z_ebf1a fw	ggatgccacacaataatcagg	84
z_ebf1a rev	acgcgggacattgtagagag	
z_ebf1b fw	tgctgccaattcaccctac	19
z_ebf1b rev	agtttgagggtaggctggtg	
z_ighμ fw	gccccaaaaacagcttctc	22
z_ ighμ rev	gaacacagttgcacgcagat	

Part III

etv6 is involved in primitive erythropoiesis in zebrafish

Abstract

ETV6 is a transcription factors that is frequently involved in chromosomal translocations in various hematological malignancies. It was shown that ETV6 is required specifically for hematopoiesis of all lineages within the bone marrow, which is the site of definitive hematopoiesis, but not in the yolk sac and fetal liver in mice, the sites of primitive hematopoiesis. Moreover, ETV6 accelerates erythroid differentiation of human and mouse leukemia cell lines and it stimulates hemoglobin synthesis in adult mice. To characterize the role of *etv6* during zebrafish development we first analyzed its expression pattern and we found that it is expressed in several hematopoietic tissues. Indeed, *etv6* is expressed in the Anterior and Posterior Lateral Mesoderm, in the Intermediate Cell Mass, in the Caudal Hematopoietic Tissue and in the Aorta-Gonad-Mesonephros region. Moreover, *etv6* loss of function determined the reduction of primitive mature erythrocytes and we found that this alteration is due to the defective differentiation of primitive erythrocytes. Interestingly, the alteration of primitive erythrocytes differentiation induced by *etv6* knockdown is, at least in part, due to the activation of Notch pathway. In fact, *etv6* loss of function caused the upregulation of the Notch effector *her6* and the treatment with the Notch pathway inhibitor DAPT partially rescued the alteration of primitive erythrocytes differentiation. Finally, *etv6* is involved, directly or indirectly, in the repression of *klf1, klf3, klf6a* and *klf17* genes, which are essential for primitive erythrocytes maturation. Thus, we show here that *etv6* is a fundamental regulator of primitive erythrocytes differentiation through the regulation of *klf* genes expression and the modulation of the Notch pathway signalling.

Introduction

ETV6 (*E-Twenty-Six Variant gene 6*), also known as *TEL* (*Translocation Ets Leukemia*), belongs to the *ETS* (*E-Twenty-Six*) gene family and encodes a transcription factor that is characterized by the C-terminal ETS domain which is responsible for the binding of DNA (Poirel *et al.,* 1997). ETV6 protein is characterized also by the N-terminal Helix-Loop-Helix (HLH) domain, also known as Pointed or Sterile Alpha Motif domain, which is involved in ETV6 homodimerization and oligomerization with other ETS proteins like Fli1 and ETV7 (Donaldson *et al.,* 1996; Kwiatkowski *et al.,* 1998; Potter *et al.,* 2000; Bohlander, 2005). It has been suggested that ETV6 acts mainly as a transcriptional repressor

consequently to the recruitment of several repressor cofactors by the HLH domain or the central region of ETV6 protein (Lopez *et al.,* 1999; Chakrabarti and Nucifora, 1999; Guidez *et al.,*2000; Boccuni *et al.,* 2003).

During mouse development *Etv6* is expressed in several tissues and organs, but its expression is higher in the fetal liver and the thymus. Moreover, *Etv6* transcript is present in various cell lines representing several blood cell lineages. Consistent with its expression in murine blood cell lines, *Etv6* is required for establishing hematopoiesis within the bone marrow but is dispensable for embryonic hematopoiesis that occur in yolk sac and fetal liver (Wang *et al.,* 1997; Wang *et al.,* 1998; Hock *et al.,* 2004). The role of *Etv6* is required also in erythropoiesis. In fact, ETV6 accelerates erythroid differentiation of human and mouse leukemia cell lines and it stimulates hemoglobin synthesis in adult mice (Waga *et al.,* 2003; Takahashi *et al.,* 2005; Eguchi-Ishimae *et al.,* 2009).

In zebrafish, primitive erythroid precursors originate from the posterior lateral plate mesoderm (PLM) around 5 somite stage. Around 18 somite stage, these precursors migrate to the intermediate cell mass (ICM). Within the ICM, erythroid progenitors differentiate in proerytrhroblasts that enter the circulation at 24-26 hpf (hours post fertilization; Detrich *et al.,* 1995). These cells continue to mature in circulation and are the only circulating red cell population through 4dpf (Weinstein *et al.,* 1996; Chen and Zon, 2009). Erythrocytes differentiation consists in cytological changes in cell size and in nuclear shape that are accompanied by 'maturational' globin switching as erythrocytes differentiate (Ransom et al., 1996; Ganis *et al.,* 2012). The members of the Kruppel-like transcription factor family are important transcriptional regulators of erythropoiesis, particularly of erythrocytes maturation, because are known to activate several erythroid-specific genes (Donze *et al.,* 1995; Hodge *et al.,* 2006; Pilon *et al.,* 2006; Gardiner *et al.,* 2007; Fu *et al.,* 2009; Palis, 2014 Xue *et al.,* 2015).

Recently, it has been reported that *etv6* is involved in both primitive and definitive hematopoiesis during zebrafish development (Rasighaemi *et al.,* 2015). We confirm here that *etv6* is expressed in tissues involved in both primitive and definitive hematopoiesis and that *etv6* is essential for primitive erythrocytes maturation. Moreover, we tried to shed light into the mechanism through which *etv6* regulates primitive erythrocytes maturation. Interestingly, we found that *etv6* is a potential modulator of the notch signaling in primitive erythrocytes. Furthermore, *etv6* is involved, directly or indirectly, in the repression of *klf1*, *klf3*, *klf6a* and *klf17* genes, which are essential for primitive erythrocytes maturation (Gardiner *et al.,* 2007; Fu *et al.,* 2009; Xue *et al.,* 2015).

Results and discussion

To characterize the *etv6* expression pattern we performed whole mount *in situ* hybridization (WISH) and we found that it is expressed in several hematopoietic tissues. Indeed, *etv6* is expressed in the anterior lateral mesoderm (ALM) and in the PLM at the 10 somite stage (Fig. 1A, B, D). Then, *etv6* transcript is present in the ICM at the 20 somite and 24 hpf stages (Fig. 1E, H). The PLM is the region where the primitive erythroid precursors are generated, whereas in the ICM they start the differentiation process (Detrich *et al.,* 1995). At 36 hpf *etv6* is expressed in the caudal hematopoietic tissue (CHT; Fig. 1L), which is a transient site of definitive hematopoiesis (Chen and Zon, 2009), and in the ventral wall of the dorsal aorta (Fig. 1L-N), which corresponds to the Aorta-Gonad-Mesonephros (AGM) region. The definitive HSCs emerge from this region, they enter circulation through the axial vein and migrate first to the CHT and then to the organs that will be the sites of adult hematopoiesis, which are the thymus and the kidney marrow, the analogous of the bone marrow in mammals (Murayama *et al.,* 2006; Jin *et al.,* 2007; Bertrand *et al.,* 2008; Kissa *et al.,* 2008; Bertrand *et al.,* 2010; Kissa and Herbomel, 2010). Besides hematopoietic tissues *etv6* is expressed also in the nervous system. In fact, *etv6* transcript is present in the neural tube at 10 and 20 somite stages (Fig. 1C, E, G). At the latter stage, *etv6* is expressed also in the cranial ganglia (Fig. 1F). Its expression in this region is maintained at 24 hpf (Fig. 1I), when *etv6* transcript is present in the brain, specifically in the mesencephalon, in the cerebellum and in the hindbrain (Fig. 1H). This expression pattern in brain is the same at 36 hpf (Fig. 1L).

To characterize the role of *etv6* in hematopoiesis, we performed *etv6* knockdown by the injection of 0,5 pmol/embryo of a translation blocking morpholino that it has been already published and validated (Roukens *et al.,* 2010). The injection of *etv6* morpholino (*etv6*-MO) caused morphological alterations that were not previously described. Specifically, *etv6* knock-down caused apoptosis in brain tissue and curved tail at 24 hpf (Fig. S1A). It is known that some morpholino molecules could elicit p53 off-target activation causing nonspecific morphological defects similar to those observed in *etv6* morphants (Robu *et al.,* 2007). We verified that *etv6*-MO causes the activation of an N-terminal truncated p53 isoform that is diagnostic for the nonspecific p53 activation (Fig. S1B; Robu *et al.,* 2007); moreover, *etv6* morphants phenotype is rescued by *p53*-MO coinjection (Fig. S1A). Consequently, we decided to coinject *etv6*-MO along with *p53*-MO in the subsequent experiments to suppress off-target effects and facilitate the study of specific loss of function phenotypes.

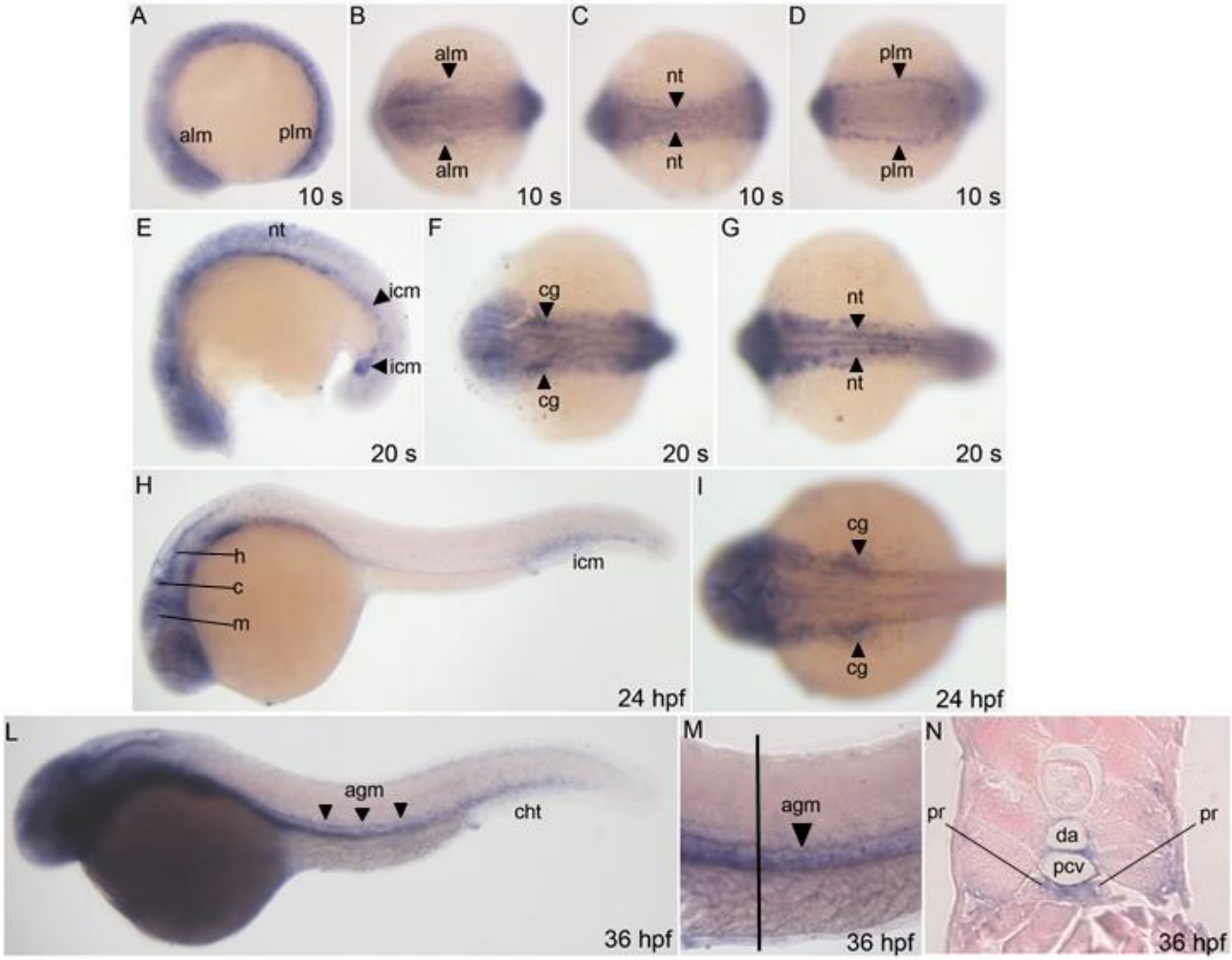

Figure 1: *etv6* is expressed in hematopoietic tissues during zebrafish development. WISH analysis showing *etv6* expression pattern from 10 somite (s) stage to 36 hpf. (A-D) At 10 somites stage *etv6* transcript is present in the anterior lateral mesoderm (alm), in the posterior lateral mesoderm (plm) and in the neural tube (nt). (E-G) At 20 somites stage *etv6* expression in the neural tube is maintained and was expressed also in the intermediate cell mass (icm) and in cranial ganglia (cg). (H, I) At 24 hpf *etv6* mRNA is still present in the icm and in cg, while it is present also in the brain, specifically, in the hindbrain (h), in the cerebellum (c) and in the mesencephalon (m). (L-N) At 36 hpf *etv6* is expressed in the aorta-gonad-mesonephros region (agm) and in the caudal hematopoietic tissue (cht). (M) Detail of the trunk of the embryo depicted in (L). (N) Transversal section of an embryo at 36 hpf showing that *etv6* hybridization signal is present in endothelial cells of the dorsal aorta (da) and of the posterior cardinal vein (pcv) as well as in the pronephros. (A, E, H, L, M) Lateral views; dorsal is up, anterior is left. (C, G, I) Dorsal views, anterior is left. (B, F) anterior views, posterior is right. (D) Posterior view, anterior is left. (N) Transversal section of the trunk; dorsal is up. Abbreviations: agm, aorta-gonad-mesonephros; alm, anterior lateral mesoderm; cht, caudal hematopoietic tissue; c, cerebellum; h, hindbrain; icm, intermediate cell mass; m, mesencephalon; plm, posterior lateral mesoderm; nt, neural tube.

Since *etv6* is involved in erythropoiesis in mammals and we found that it is expressed in tissues related to primitive erythropoiesis (Waga *et al.,* 2003; Takahashi *et al.,* 2005; Eguchi-Ishimae *et al.,* 2009), we decided to investigate if *etv6* is involved in primitive erythropoiesis in zebrafish. Thus, we analyzed the hemoglobin content of *etv6* morphants by whole embryo o-dianisidine staining in order to assess the presence of primitive differentiated erythrocytes. Primitive mature erythrocytes appeared reduced in the 90% (n=41) of *etv6*-atg-MO/*p53*-MO coinjected embryos at 48 hpf compared to control embryos (Fig. 2A), thus indicating an alteration of primitive erythropoiesis. However, we did not observe circulatory defects nor a decrease of circulating blood cells at 48 hpf in morphant embryos (data not shown), suggesting that *etv6* loss of function did not cause the reduction of primitive erythrocyte precursors. To further verify that the production of primitive erythrocyte precursors is not impaired in *etv6* morphants, we analyzed the expression of some primitive erythrocytes markers at 24hpf, at the onset of primitive erythrocytes differentiation (Detrich *et al.,* 1995). Specifically, we analyzed the expression of: *gata1*, the master regulator of erythropoiesis (Fujiwara *et al.,* 1996; Galloway *et al.,* 2005; Rhodes *et al.,* 2005), *alas2*, which encodes an enzyme involved in hemoglobin synthesis (Brownlie *et al.,* 1998), and the embryonic globin genes *hemoglobin beta embryonic 3* (*hbbe3*) and *hbbe1* (Ganis *et al.,* 2012). WISH and qRT-PCR assays showed that the expression of these markers is comparable between morphants and control embryos at 24 hpf (Fig. 2B, C), thus confirming that primitive erythrocytes precursors are correctly generated. Starting from about 24-26 hpf erythroid progenitors differentiate in proerytrhroblasts and enter the circulation continuing the maturation process (Detrich *et al.,* 1995; Weinstein *et al.,* 1996; Chen and Zon, 2009). At 48 hpf *hbbe3* and *gata1* must be downregulated to allow primitive erythrocytes differentiation. Therefore, we analyzed the expression of these genes by WISH and qRT-PCR assays at 48 hpf to verify the proceeding of the maturation process. WISH assays showed that *hbbe3* and *gata1* are correctly downregulated in control embryos whereas they are still up-regulated in the 96% (n=25) and 89,47% (n=17) of *etv6* morphant embryos respectively (Fig. 2D). We confirmed also by RT-PCR analysis that *hbbe3* (FC = 2,17 $p < 0,001$) and *gata1* (FC = 1,49 $p < 0,01$) are upregulated in *etv6* morphants, while *alas2* is downregulated (FC = 0,46 $p < 0,001$; Fig. 2E). Overall, our findings strongly suggest that *etv6* is not involved in the emergence of primitive erythrocytes precursors, whereas it is essential for primitive erythrocytes differentiation in zebrafish.

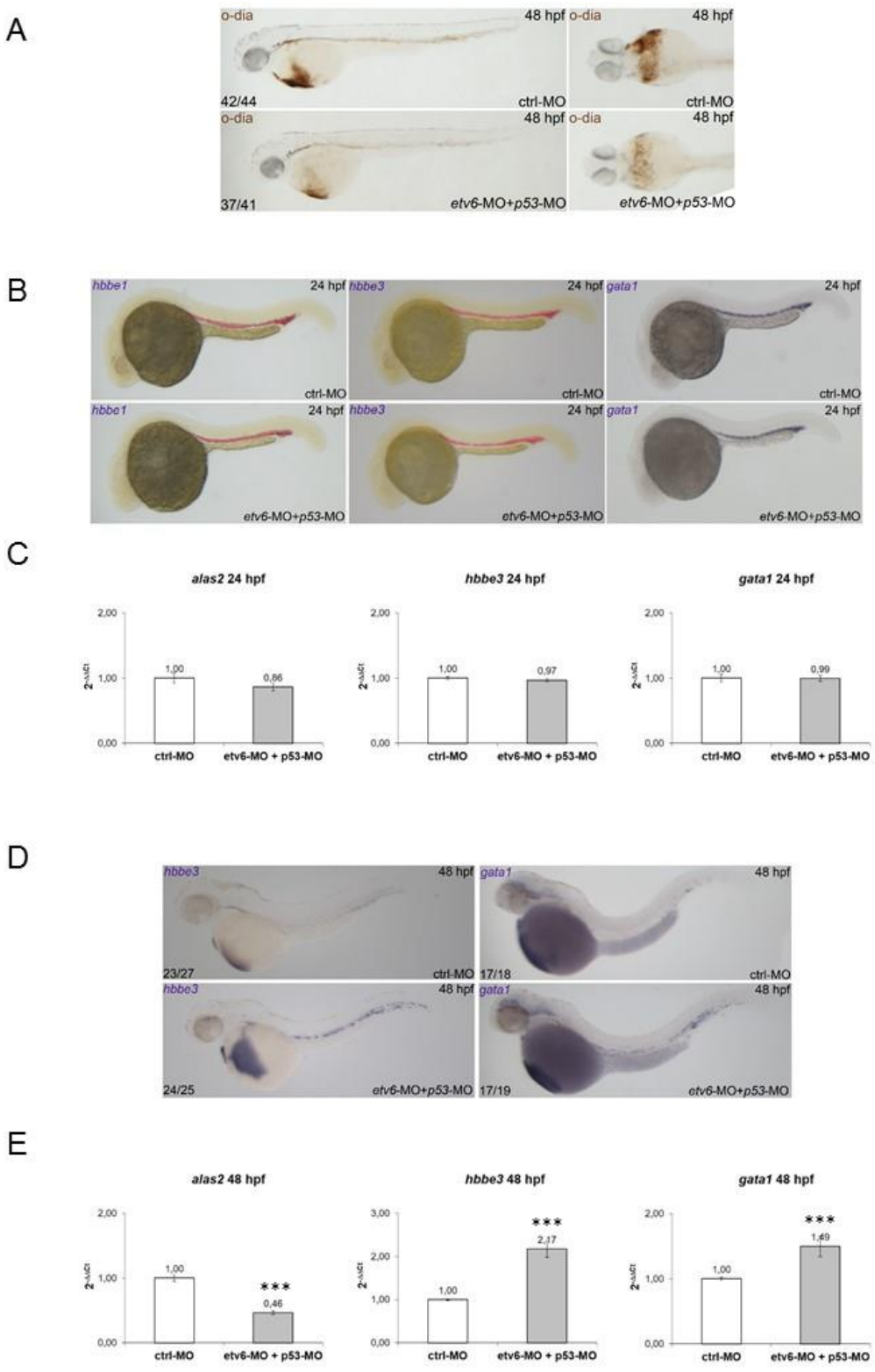

Figure 2: Primitive erythrocytes differentiation is impaired in *etv6* morphants. Legend in the next page.

Figure 2: Primitive erythrocytes differentiation is impaired in *etv6* morphants. (A) O-dianisidine staining highlights the reduction of hemoglobin in *etv6* morphants at 48 hpf. (B, C) The expression of the primitive erythrocytes markers at 24 hpf, at the onset of erythrocytes differentiation, is comparable between controls and *etv6* morphants suggesting that primitive erythrocyte precursors are correctly generated. (B) WISH assays of *hbbe1*, *hbbe3* and *gata1* genes at 24 hpf. (C) qRT-PCR assays *alas2*, *hbbe3* and *gata1* at 24 hpf. (D, E) The expression of *hbbe3* and *gata1* is abnormally upregulated in *etv6* morphants at 48 hpf, while it is correctly downregulated in control embryos. (D) WISH assays of *hbbe3* and *gata1* genes at 48 hpf. (E) qRT-PCR assays *alas2*, *hbbe3* and *gata1* at 24 hpf; *alas2* is downregulated in *etv6* morphants. (A left, B, D) Lateral views; dorsal is up, anterior is left. (A right) ventral views, anterior is left. (E) *t* test: * = $p < 0,05$; ** = $p < 0,01$; *** = $p < 0,001$.

A previous paper of our lab showed that the alteration of primitive erythrocytes differentiation can be caused by the increased activation of the Notch pathway leading to the upregulation of the Notch downstream target *her6* in the PLM at about 10 somite stage (Bresciani *et al.,* 2010). *her6* is the zebrafish homologue of the mammalian gene *HES1*, which encodes a transcription factor that interacts with GATA1 both *in vivo* and *in vitro* and can inhibit erythroid/megakaryocytic differentiation by suppressing GATA1 activity (Ishiko *et al.,* 2005). We therefore hypothesized that the alteration of primitive erythrocytes differentiation observed in *etv6* morphants could be caused by the overexpression of *her6*. Hence, we analyzed *her6* expression by WISH assays and we found that its hybridization signal is upregulated and expanded in 85,19% (n=27) of *etv6* morphants compared to control embryos (Fig. 3A). To confirm that the alteration of primitive erythrocytes differentiation is caused by the increased activity of the Notch pathway, we tried to rescue the defective maturation observed at 48 hpf by treating *etv6* morphants with the Notch inhibitor DAPT starting from about 10 somites. O-dianisidine staining showed that the alteration of primitive erythrocytes differentiation is partially rescued in the 75% (n=40) of *etv6* morphants treated with 100μM DAPT compared to untreated *etv6*-atg-MO injected embryos (Fig. 3B). Thus, the defective primitive erythrocytes maturation in *etv6* morphants is, at least in part, caused by the increased activity of the Notch pathway. Furthermore, our results indicate *etv6* as a potential modulator of the Notch pathway signaling in primitive erythrocytes.

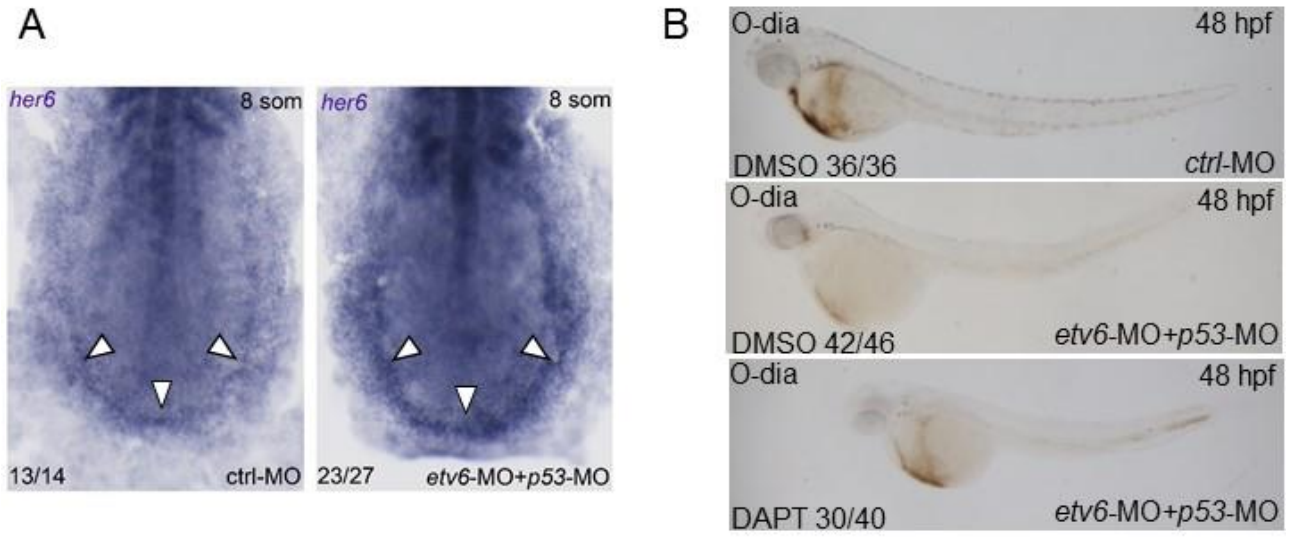

Figure 3: *etv6* is involved in the modulation of the Notch pathway. (A) Flat mount preparation of WISH assay showing the upregulation and the expansion of *her6* expression in the posterior lateral mesoderm (arrowhead) of *etv6* morphants at 8 somites. The detail of the PLM is shown. Dorsal view, anterior is up (B) The decreased o-dianisidine staining observed in *etv6* morphants is partially rescued by the treatment with 100μM of the notch pathway inhibitor DAPT. Lateral views, anterior is left, dorsal is up.

Recently, it has been reported that *ETV6* mutations can alter the expression of *KLF6* (Zhang *et al.*, 2015). Since the zebrafish *klf6a* gene, along with *klf1*, *klf3* and *klf17* are involved in the differentiation of primitive erythrocytes (Gardiner *et al.*, 2007; Fu *et al.*, 2009; Xue *et al.*, 2015), we thus decided to test by qRT-PCR analysis if *etv6* knockdown can cause the alteration of these genes at 24 hpf and 48 hpf. At 24 hpf the expression of *klf3* (FC = 1,63 $p < 0,001$) and *klf17* (FC = 1,61 $p < 0,01$) is significantly upregulated in *etv6* morphants, while the expression of *klf1* and *klf6a* is comparable between control and morphant embryos (Fig. 4). At 48 hpf *klf3* (FC = 1,28 $p < 0,05$) and *klf6a* (FC = 1,37 $p < 0,05$) are slightly but statistically significant upregulated, whereas *klf1* (FC = 4,64 $p < 0,001$) and *klf17* (FC = 11,60 $p < 0,01$) are strongly upregulated in *etv6* morphants compared to control embryos (Fig. 4).

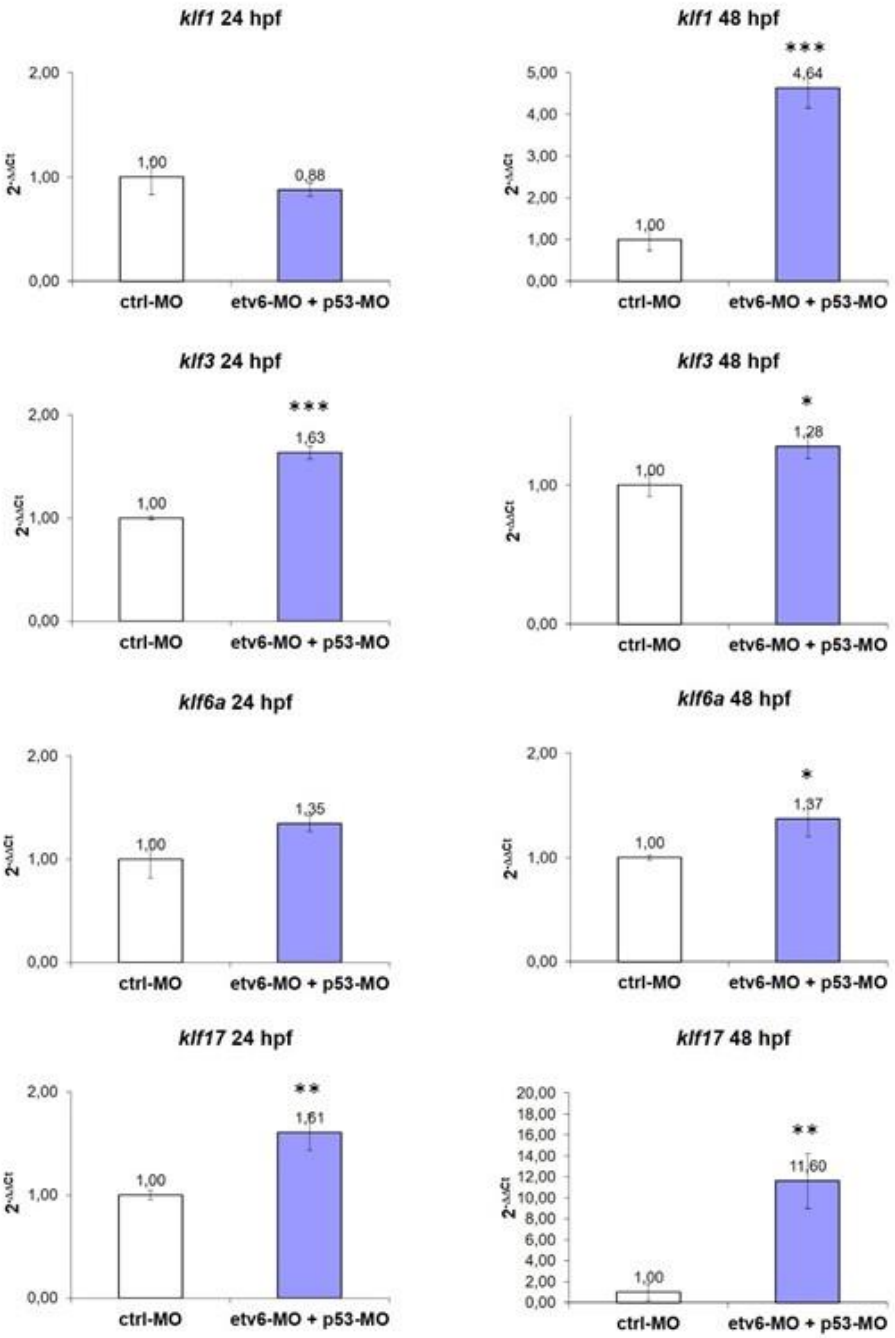

Figure 4: *etv6* knockdown causes the upregulation of *klf* genes. At 24 hpf only *klf3* and *klf17* are upregulated in *etv6*-MO injected embryos. However, at 48 hpf all *klf* genes analysed are upregulated in *etv6* morphants and among them *klf1* and *klf17* are the most strongly activated. *t* test: * = $p < 0,05$; ** = $p < 0,01$; *** = $p < 0,001$.

These results are consistent with the roles of *klf* genes in zebrafish primitive erythrocytes maturation. In fact, *klf3*, *klf6a* and *klf17* knockdown determined the downregulation of *hbbe3* (Gardiner *et al.*, 2007; Xue *et al.*, 2015), and we report that *etv6* loss of function induced the upregulation of both *klf* genes and *hbbe3*. Moreover, *klf1* in zebrafish is known to be involved in primitive erythropoiesis regulating *α globin* gene, but in mammals KLF1 primarily regulates the adult *β globin* gene. Besides embryonic *globin* genes, Klf transcription factors control also the expression of multiple erythroid-specific genes, such as those involved in heme synthesis (Donze *et al.*, 1995; Hodge *et al.*, 2006; Pilon *et al.*, 2006; Gardiner *et al.*, 2007; Fu *et al.*, 2009; Xue *et al.*, 2015). Thus, *etv6* could potentially influence several processes of erythrocytes differentiation via the modulation of *klf* genes expression. The notion that ETV6 is mainly known as a transcriptional repressor (Lopez *et al.*, 1999; Chakrabarti and Nucifora, 1999; Guidez *et al.*,2000), together with the upregulation of *klf* genes consequently to *etv6* knockdown, lead us to speculate that Etv6 could directly repress *klf* genes transcription, maintaining the correct level of *klf* genes expression during primitive erythropoiesis. However, further experiments are needed to verify the direct interaction of Etv6 with the promoters of *klf* genes.

Overall, our findings highlight the key role of Etv6 in the differentiation of primitive erythrocytes, exerted through the regulation of *klf* genes expression and the modulation of the Notch pathway signalling.

Materials and methods

Zebrafish lines and maintenance

Zebrafish (*Danio rerio*) embryos were raised and maintained under standard conditions and national guidelines (Italian decree 4th March 2014, n.26). All experimental procedures were approved by IACUC (Institutional Animal Care and Use Committee). Zebrafish AB wild-type strains were obtained from the Wilson lab, University College London, London, United Kingdom. For in vivo observations and imaging, embryos beyond 24 hpf were washed, dechorionated and anaesthetized with 0.016% tricaine (ethyl 3-aminobenzoate methanesulfonate salt; Sigma-Aldrich) before observations and picture acquisition. Embryos were staged according to morphological criteria (Kimmel *et al.*, 1995).

Whole mount *in situ* hybridization, embryo sectioning, o-dianisidine staining and imaging

Whole mount *in situ* hybridizations were carried out as described (Thisse and Thisse, 2007). The riboprobes were synthesized using the Ambion® MAXIscript® SP6/T7 *In Vitro* Transcription Kit (Thermo Fisher Scientific). We synthesized probes as described in the following papers: *gata1* (Detrich *et al.,* 1995), *hbbe1* and *hbbe3* (Ganis *et al.,* 2012), *her6* (Pasini *et al.,* 2001). To synthesize *etv6* probe we amplified 877 bp of the coding sequence that we subcloned in the pGEM-T easy vector (Promega). The construct was then digested to synthesize antisense and sense probes. To amplify the sequence used for the synthesis of the probes we used the following primers:

etv6-probe-fw	5'-	CGCGAGGGGAACATTATCCA	-3'
etv6-probe-rev	5'-	AGGAGATACAGACAGCGGGT	-3'

Images of stained embryos were taken on a Leica MZFLIII epifluorescence stereomicroscope equipped with a DFC 480 digital camera and LAS Leica imaging software (Leica, Wetzlar, Germany). Some of the hybridized embryos were then embedded in paraffin (Paraplast plus, Bio Optica) and sectioned (8μm) on a microtome (Leitz 1516). The slides were mounted with Eukitt (Bio Optica) and all sections were observed using a Leica DM6000B microscope equipped with a DFC 480 digital camera and LAS Leica imaging software (Leica, Wetzlar, Germany). O-dianisidine staining was carried out as described (Detrich et al., 1995)

Real-time quantitative PCR assay and semi-quantitative RT-PCR

Total RNA was isolated from embryos at different developmental stages using the SV Total RNA isolation System kit (Promega, Madison, Wisconsin, USA). After treatment with DNase I RNase-free (Roche, Basel, Switzerland), 1 μg of RNA was reverse-transcribed using the ImProm-II™ Reverse Transcription System (Promega) according to manufacturer's instructions. Real-time analysis was performed on Light Cycler 480II with Universal Probe Master system (Roche Diagnostics; F. Hoffmann-La Roche Ltd.). Primers and probes were selected according to the Software Probe Finder (Roche Diagnostics) and are reported in table below. Data were expressed using the comparative ΔΔCt method, using *rpl8* gene as reference and *ctrl*-MO or *GFP* mRNA injected embryos as standardization control; both t test and SD values refer to triplicates of a single experiment and N=3 biological independent experiments were performed for each gene.

To assess the non-specific activation of p53, semi-quantitative RT-PCR were performed with cDNA obtained from *etv6* morphants at 24hpf. *p53* and *Δ113-p53* were subjected to 20 or 25 amplification cycles using primers reported in (Robu *et al.*, 2007).

Gene name	**Primer sequence**	**Probe number**
z_rpl8 fw	tcaagggaatcgtgaaggac	82
z_rpl8 rev	gggtcacgaaacatcacctt	
z_alas2 fw	aaaagctgctcaatcctctga	7
z_alas2 rev	tgccgctttggctctttat	
z_gata1 fw	aacgacatcttcaatactacacttgc	82
z_gata1 rev	ggacacccaacgagaagg	
z_hbbe3 fw	aagtcgacccaggcaattt	29
z_hbbe3 rev	tgcagtcatccagattaagacc	
z_klf1 fw	acggatggctgtgactcaa	8
z_klf1 rev	cagatccagaaagccttttca	
z_klf3 fw	gctgagcccgtcactcac	18
z_klf3 rev	acgaagggtgtcagaacctg	
z_klf6a fw	ttgccatcgcttgaagaata	78
z_klf6a rev	gtaaggctcgctctgcaaat	
z_klf17 fw	ccacattttagacgagaagcagt	19
z_klf17 rev	tccacctcaataaggggtctc	

Morpholino microinjection

Antisense morpholinos were purchased from Gene Tools (LLC, Philomath, OR). Morpholinos were diluted in Danieau solution (Nasevicius and Ekker, 2000) and injected into 1-to-2 cell-stage embryos using Eppendorf FemtoJet Micromanipulator 5171. Rhodamine dextran (Molecular Probes, Life technology) was co-injected as dye tracer. *etv6* knockdown was performed injecting 0,5pmol/embryo of a translation blocking MO (*etv6*-MO 5'-CATGTCTCGTTGAAATTCAGGAAGT-3') previously described (Roukens *et al.*, 2010). A standard control oligo (ctrl-MO, 5'-CCTCTTACCTCAGTTACAATTTATA-3', against human *β-globin* gene) with no target in zebrafish embryos, was also used, to check for non-specific effects due to the injection procedure. Co-injection of *etv6*-MO with 0,75 pmol/embryo p53-MO was performed to abolish aspecific effects (Robu et al., 2007).

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Supplementary figure S1

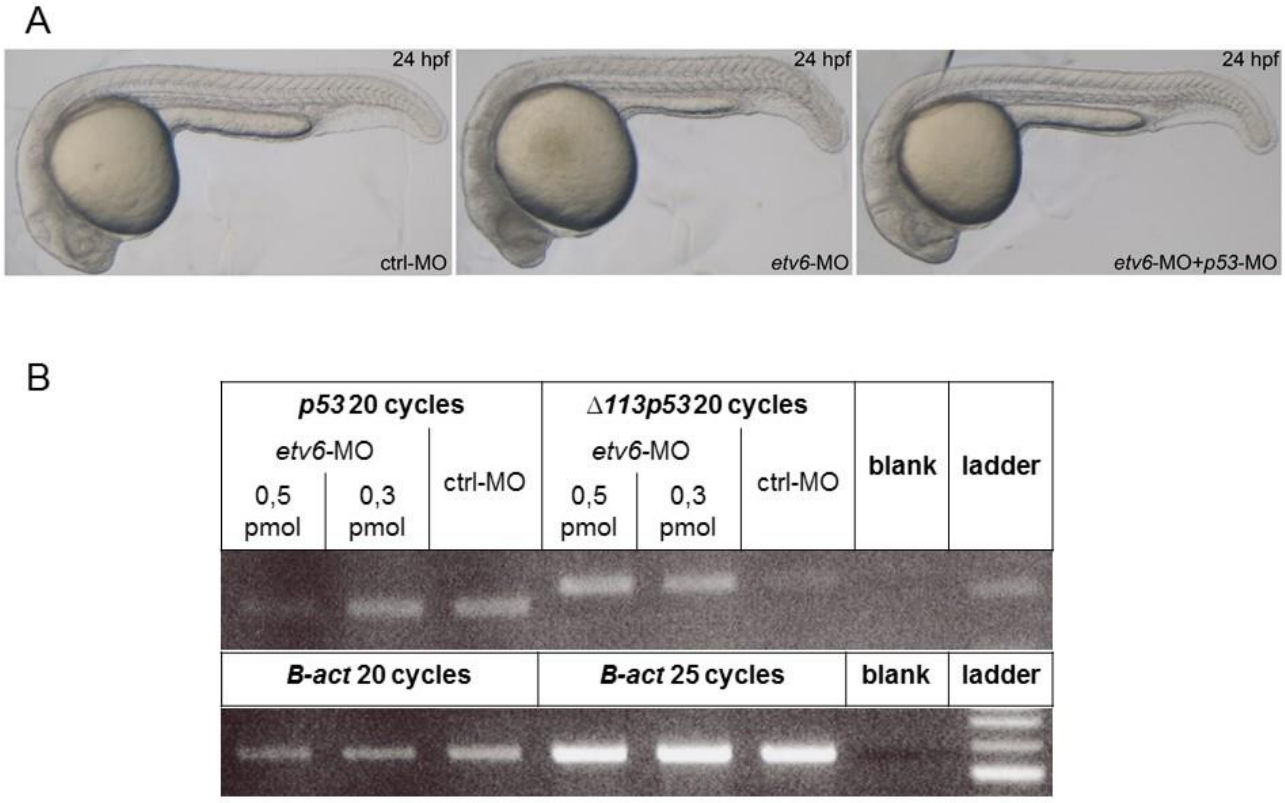

Figure S1: *etv6*-MO induces the non-specific activation of *Δ113-p53*. (A) The injection of 0,5 pmol/embryo of *etv6*-MO causes apoptosis in brain tissue and curved tail at 24 hpf. These alterations are rescued by the co-injection of 0,75 pmol/embryo of *p53*-MO along with *etv6*-MO. (B) Semi-quantitative RT-PCR performed at 24 hpf showing that *etv6*-MO injection induces the upregulation of *Δ113-p53*, encoding a truncated p53 isoform which is considered diagnostic for non-specific p53 activation caused by some morpholino molecules (Robu *et al.*, 2007). Also the semi-quantitative RT-PCRs of *p53* and *βactin* are reported. *βactin* amplification at 25 cycles is shown to compare the saturation level.

The Coiled-Coil Domain Containing 80 (*ccdc80*) gene regulates *gadd45beta2* expression in the developing somites of zebrafish as a new player of the hedgehog pathway

During my Ph.D., I have been also partecipated in the characterization of *ccdc80* gene function in zebrafish development as side project. The *Coiled-Coil-Domain Containing 80* (*Ccdc80*) gene, also named *DRO1* in human (*Down-Regulated by Oncogene 1*), *URB* in mouse (*Up-Regulated in BRS-3 deficient mice*), *CL2* in rat (*Clone 2*), and *equarin* in chicken, has been recently suggested to be involved in different functions among vertebrates. *Ccdc80* was first isolated in mice, where it is up-regulated in adipose tissue of obese BRS-3-deficient animals [1]. Moreover, *Ccdc80* is highly expressed in mice white adipose tissue and its silencing inhibits adipocytes differentiation [2], suggesting a role in the regulation of body weight and energy metabolism. *Ccdc80* is also expressed in mouse developing cartilage, suggesting a role during skeletogenesis [3]

Human *CCDC80* is almost ubiquitously expressed, with the highest levels in heart and skeletal muscles [4,5]. Furthermore, human *CCDC80* can be considered a potential tumor suppressor gene [6]. In fact, it is strikingly down-regulated in thyroid neoplastic cell lines and tissues, as well as in colon and pancreatic cancer cell lines and in most colorectal cancer specimens [7], while its ectopic expression in these cell lines results in substantial inhibition of growth properties. The CCDC80 protein is highly conserved among vertebrates, and contains multiple signals of cellular compartmentalization and post-translational modifications. In particular, it has a N-terminus leader peptide for extracellular export and many nuclear localization signals [6]. In different studies, the CCDC80 protein has been identified in a N-glycosylated form and was suggested to be secreted. Rat, mouse and human CCDC80 show three PDUDES domains (Procaryotes- DRO1-URB-DRS-Equarin-SRPUL) which in human are correlated with a tumor suppressor role [8]. We have previously reported that in zebrafish there are three homologs of the human *CCDC80*gene [9]. The zebrafish *ccdc80-l1* gene is expressed in muscular tissues, specifically in adaxial cells and muscle pioneers, which are both involved in axon pathfinding of motoneurons. Despite somitogenesis and myogenesis processes occurred properly in *ccdc80-l1* knocked-down embryos, *ccdc80-l1* loss of function impaired the proper axonal pathfinding, especially in ventral axons guidance. Furthermore, the analysis of *ccdc80-l1* upstreamregulation revealed that the Hedgehog pathway

modulates its expression in territories involved in axonal guidance (Brusegan *et al.,*2012). Herein we show that zebrafish *ccdc80* paralogue is strongly expressed in the notochord but it is not required for the correct formation of this structure. However, loss and gain of function experiments suggests that *ccdc80* is involved in somitogenesis process as a downstream effector of the Hedgehog pathway and as an upstream regulator of *gadd45b2*.

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Journal of Cellular Physiology

The Coiled-Coil Domain Containing 80 (*ccdc80*) Gene Regulates *gadd45β2* Expression in the Developing Somites of Zebrafish as a New Player of the *Hedgehog* Pathway

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The *Coiled-Coil Domain Containing 80 (CCDC80)* gene has been identified as strongly induced in rat thyroid PC CL3 cells immortalized by the adenoviral E1A gene. In human, *CCDC80* is a potential oncosoppressor due to its down-regulation in several tumor cell lines and tissues and it is expressed in almost all tissues. *CCDC80* has homologous in mouse, chicken, and zebrafish. We cloned the zebrafish *ccdc80* and analyzed its expression and function during embryonic development. The in-silico translated zebrafish protein shares high similarity with its mammalian homologous, with nuclear localization signals and a signal peptide. Gene expression analysis demonstrates that zebrafish *ccdc80* is maternally and zygotically expressed throughout the development. In particular, *ccdc80* is strongly expressed in the notochord and it is under the regulation of the *Hedgehog* pathway. In this work we investigated the functional effects of *ccdc80*-loss-of-function during embryonic development and verified its interaction with *gadd45β2* in somitogenesis.

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In vertebrates muscles develop from the somites, bilateral pairs of mesodermal segments flanking the notochord (Stemple, 2005). In zebrafish (*Danio rerio*), somitogenesis occurs between 10.5 hours post fertilization (10.5 hpf) and 24 hpf (Stickney et al., 2000). Somites are created sequentially in an anterior to posterior sequence, concomitantly with the posterior growth of the trunk and tail (Holley, 2007). Cells in the presomitic mesoderm (PSM) alter their adhesive properties and undergo mesenchymal to epithelial transitions, forming somite pairs every 20–30 min until a total of about 30 somites (Stickney et al., 2000). A molecular clock has been proposed for the regulation of this reiterative process. The clock causes cells in the PSM to undergo cyclical activation and repression of several Notch pathway genes, such as *her* genes, a family of transcriptional repressors (Oates et al., 2005; Echeverri and Oates, 2007). Each cell in the PSM periodically makes and degrades the products of these genes, in a synchronous manner with its neighbors. Soon after segmentation, morphologically distinct somites are created via the generation of a boundary between anterior and posterior somite halves. Many genes behave as segment-polarity genes; they are localized in the anterior or posterior half of the somite. For example, in zebrafish the two bHLH (basic-helix-loop-helix) transcription

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factors *mesp-a* and *mesp-b* are required for the specification of anterior and posterior identity within each segment (Sawada et al., 2000). As somite polarity is established, morphological segmentation occurs. Mutations that affect clock functions also perturb segment polarity, with markers of anterior and posterior half of the somite generally expressed in a disorganized manner throughout the somitic mesoderm (Durbin et al., 2000). In zebrafish the GADD45 family members *gadd45β1* and *gadd45β2* are expressed as paired stripes adjacent to the neural tube in the anterior PSM region where presomitic cells mature. The knock-down and over-expression of *gadd45*β genes caused somite defects (Kawahara et al., 2005).

The Coiled-Coil-Domain Containing 80 (Ccdc80) gene, also named DRO1 (Down-regulated by Oncogene 1) and SSG1 (Steroid Sensitive Gene 1) in human, Urb in mouse (Up-regulated in BRS-3 deficient mice), CI2 (Clone 2) in rat and EQUARIN in chicken, has been recently suggested to be involved in different functions among vertebrates. Ccdc80 was first isolated in mice (Aoki et al., 2002), where it is up-regulated in adipose tissue of obese BRS-3-deficient animals and it was suggested to play a role in the regulation of body weight and energy metabolism (Tremblay et al., 2009). Moreover, murine Ccdc80 is expressed in the developing cartilage, suggesting a role during skeletogenesis (Liu et al., 2004). In chickens, two alternative splicing forms of CCDC80 are described: S- and L-EQUARIN. During embryogenesis, both transcripts are detected in the developing lens, with a high dorsal to ventral gradient. Microinjection of equarin mRNAs in xenopus embryos induced abnormal eye development, suggesting its involvement in eye formation (Mu et al., 2003). Human CCDC80 is almost ubiquitously expressed, with the highest levels in heart and skeletal muscle (Visconti et al., 2003). Studies of expression profiling show that CCDC80 is expressed also in skeletal myotubes during normal differentiation of skeletal muscles (Raymond et al., 2010), and in muscles from patients with Duchenne dystrophy (Tseng et al., 2002; Haslett et al., 2003). In skeletal myotubes its expression is elicited by pyruvate (Wilson et al., 2007) and suppressed by starvation (Stevenson et al., 2005), suggesting a possible role for CCDC80 as a cell differentiation protein both in cultured cells and muscle tissues undergoing regeneration or remodeling.

Human *CCDC80* is also considered a potential tumor suppressor gene (Visconti et al., 2003; Ferraro et al., 2013), as it is strikingly down-regulated in thyroid neoplastic cell lines and tissues, in colon and pancreatic cancer cell lines and in most colorectal cancer specimens (Bommer et al., 2005). Further evidences of its involvement in tumors came from observation of *CCDC80* significant down-regulation in transgenic mouse mammary epithelial cells over-expressing the oncogene *AIB1* (Ferragud et al., 2011). The encoded CCDC80 protein is highly conserved among vertebrates, and contains multiple signals of cellular compartmentalization and post-translational modifications. In particular, it has a N-terminus leader peptide for extracellular export and many nuclear localization signals (Visconti et al., 2003). Moreover, the CCDC80 protein has been identified in a N-glycosylated secreted form and may be secreted. Studies of rat Ccdc80 cellular localization resulted in two different sub-cellular localization patterns: juxtanuclear, indicating a Golgian localization, and nuclear-cytoplasmic (Visconti et al., 2003; Ferraro et al., 2013). Indeed, human CCDC80 protein has been identified both in a N-glycosylated secreted form (Bommer et al., 2005) and in a nuclear form (Wang et al., 2013).

Human CCDC80 shows high sequence identity with its homologous in rat (84%), mouse (83%), and chicken (66%). In all species studied, the protein is highly basic and shows a lysine- and a threonine-rich region. Moreover, three P-DUDES domains (Procaryotes-*DRO1*-*URB*-*DRS*-*Equarin*-*SRPUL*) are present. In human, P-DUDES are correlated with a tumor suppressor role and are found in single copy also in SRPX and SRPX2 proteins, which are involved in tumor suppression and progression respectively (Pawlowski et al., 2010).

In this work, we investigated the function of *ccdc80* in-vivo, during the embryonic development of zebrafish by means of loss- and gain-of-function experiments. Our results suggest a new functional role for the zebrafish homologous *ccdc80* during somitogenesis as a downstream effector of the *Hedgehog* signaling (*Hh*), and as an upstream regulator of *gadd45β2*.

Materials and Methods

Animals

Breeding zebrafish were maintained at 28°C on a 14 h light/10 h dark cycle. Embryos were collected by natural spawning, staged according to Kimmel and colleagues (Kimmel et al., 1995) and raised at 28°C in fish water (Instant Ocean, 0,1% Methylene Blue) in Petri dishes, according to established techniques, in agreement with EU Directive 2010/63/EU for animal welfare. We express the embryonic ages in somites (s) and hours post fertilization (hpf). AB strains were obtained from the Wilson lab (University College of London, London, United Kingdom).

Isolation and cloning of the zebrafish *ccdc80* gene

The putative zebrafish *ccdc80* ORF sequence was identified through in-silico search of the ENSEMBL zebrafish assembly version 9 (Zv9) using rat *Ccdc80 (CI2)* gene sequence previously described (Visconti et al., 2003). The zebrafish putative ORF was fully contained in the RP71–20I5 clone, linkage group 9 (GenBank Accession Number AL590146). The presumptive ORF of the zebrafish *ccdc80* was amplified by means of reverse transcription polymerase chain reaction (RT-PCR) performed on total RNA isolated from adult fish. The *ccdc80* entire coding sequence was cloned in the pCS2+ vector with the following primers:

*ccdc80_* sense: 5′-TAGAATTCGCCACCATGAGGGCAC-3′; *ccdc80_* antisense: 5′-ATAGAGCTCGAGCTAATATCC ATAACCCTGATGATAAC-3′. Cloning of the 5′ and 3′ UTR (Untranslated Region) of *ccdc80* gene was performed using the 5′/3′ RACE kit, 2nd Generation (Roche) following manufacturer's instructions, on the basis of the RP71–20I5 clone.

Following primers for the 5′ end and for the 3′ end cloning were used:

5′_*ccdc80*_sense: 5′-GCGAATTCGATTTGTCTTTGTTAA AAACTAT–3′, 5′_*ccdc80*_antisense: 5′-GACGCCAGTAG TTTGGAGAACC-3′; 3′_*ccdc80*_sense: 5′–GGCCATCCA GCAGTCTCTAGG-3′, 3′_*ccdc80*_antisense 5′–TAAGGA TCCGACCACGCGTATCGATGTCGAC-3′.

Primers for RACE reactions were designed using the Primer3 software program on the basis of the RP71–20I5 clone sequence.

Analysis of the zebrafish Ccdc80-encoded protein

Physical-chemical properties of the zebrafish Ccdc80 protein were in-silico deducted with the software program Compute pI/Mw (Gasteiger et al., 2003). The putative sub-cellular localization of the zebrafish Ccdc80 protein was predicted using the software program WoLF PSORT (Horton et al., 2007). Analysis of post-translational glycosylation sites were performed using the available tools on the web proteomics server Expasy, http://expasy.org/tools/#proteome (Gupta and Brunak, 2002; Gasteiger et al., 2003; Julenius et al., 2005). The presence of common motifs in the Ccdc80 protein was analyzed with the MEME Suite Motif-based sequence analysis tools (version 4.6.0) http://meme.nbcr.net (Bailey and Elkan, 1994; Bailey and Gribskov, 1998). Alignment of the three obtained domains was performed with the software program ClustalW2, http://www.ebi.ac.uk/Tools/msa/clustalw2/ (Chenna et al., 2003; Larkin et al., 2007).

RNA isolation

For extraction of total RNA, zebrafish embryos at different developmental stages were snap frozen in liquid nitrogen and stored at −80°C until further processing. Organs were dissected under a stereomicroscope, frozen in liquid nitrogen and stored at −80°C until further processing. Frozen specimens were homogenized in TRIZOL (Invitrogen, Carlsbad, CA) and RNA was then purified according to standard protocols.

Semi-quantitative RT-PCR

0.5 μg of total RNA was used as a template for a reverse transcription reaction with random primers and AMW-Reverse Transcriptase (Roche Diagnostics Co, Indianapolis, IN). The obtained cDNA (diluted 1:10) was used in a 50 μl PCR reaction (Taq DNA Polymerase, Eppendorf), which was stopped in the exponential phase (25 cycles). To normalize, the *elongation factor 1 alpha* (*ef1α*) gene was also amplified in a parallel semi-quantitative PCR.

Following primers were used:

*ccdc80*_sense: 5′-CCAGGATCTCATCATGGAGC–3′;

*ccdc80*_antisense: 5′-GACCAGCTTCAGCACGGACA-3′;

*ef1α*_sense: 5′-GGTACTTCTCAGGCTGACTGT–3′;

*ef1α*_antisense: 5′-CAGACTTGACCTCAGTGGTTA–3′.

10 μl of each PCR reaction were loaded on an agarose gel and blotted on a nylon Zeta-Probe GT membrane (Biorad, Hercules, CA), hybridized with cloned DNA probes covering the full-length ORF of the genes labeled with [$\alpha-^{32}$P]dCTP (Random Primed DNA Labeling Kit, Roche, Roche Diagnostics Co, Indianapolis, IN). After hybridization, blots were exposed to a T-MAT G/RA (Kodak) film for 20 min. Band densitometry was performed with the Quantity One software program, Bio-Rad Laboratories, Inc.

Injections

Injections were carried out on 1- to 2-cell stage embryos; when necessary the dye tracer rhodamine dextran (Molecular Probes, Eugene, OR) was also co-injected. To block *ccdc80* mRNA translation, an ATG- (*ccdc80*-MO1) and a pre-ATG-*ccdc80*-MO (*ccdc80*-MO2) targeting morpholinos were synthesized (Gene Tools, LLC, Philomath, OR):

ccdc80-MO1: 5′-AACCAAGCATATACCGTGCCCTCAT-3′; *ccdc80*-MO2: 5′-GCCCTCATGTTTGAACAATTTAATA-3′. and used at the concentration of 1,5 pmole/embryo (12 ng) in 1× Danieau buffer (pH 7,6) as previously reported (Nasevicius and Ekker, 2000). As a control for unspecific effects, we injected a standard control morpholino oligonucleotide that has no target in zebrafish (ctrl-MO, Gene Tools). A *ccdc80* 5′-mismatch morpholino (*ccdc80*-mismatch-MO1, Gene Tools), designed against the same region of *ccdc80*-MO1 was used as the control for unspecific effect.

ccdc80-mismatch-MO1 5′-AAgCAAcCATATAgCGT GCgCTgAT-3′.

The in-vivo test of the specificity was carried out as described in Brusegan and colleagues (Brusegan et al., 2012). In brief: 300 pg/embryo of the pCS2 + -*ccdc80*-MO1-GFP sensor plasmid have been injected alone or co-injected with 12 ng/embryo of *ccdc80*-MO1. The presence/absence of the GFP signal has been monitored under a fluorescent microscope at 20 s stage. *ccdc80*-MO1 cDNA fragments inserted in the *BamHI* site were obtained using the following complementary oligos:

ccdc80-MO_sense: 5′ *gatc*ATGAGGGCACGGTATATGCT TGGTTCA 3′; *ccdc80*-MO_antisense: 5′ *gatc*TGAACCAAGC ATATACCGTGCCCTCAT3′.

For the rescue of the *ccdc80*-MO1 phenotype, 12 ng of *ccdc80*-MO1 were injected together with 300 pg/embryo of *ccdc80* full length mRNA. *gadd45β2* mRNA was synthesized from plasmid kindly provided by Igor Dawid (Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institute of Health, Bethesda, MD). *shh* mRNA has been kindly provided by Paolo Sordino (Laboratory of Cellular and Developmental Biology, Stazione Zoologica Anton Dohrn, Naples, Italy). In over-expression experiments we have used 300 pg/embryo of *ccdc80* mRNA, 200 pg/embryo of *gadd45β2* mRNA and 300 pg/embryo of *shh* mRNA respectively. The control for unspecific effects of over-expression experiments was performed with the parallel injection of the same amount of *gfp* mRNA.

In-situ hybridization and histological analysis

Whole mount In-Situ Hybridization (WISH) experiments were carried out as described by Thisse and colleagues (Thisse et al., 1993). Embryos were fixed for 2 h in 4% paraformaldehyde/phosphate buffered saline, then rinsed with PBS-Tween, dehydrated in 100% methanol and stored at −20°C until processed for WISH (Jowett and Lettice, 1994). Antisense riboprobes were previously in-vitro labelled with modified nucleotides (digoxigenin, Roche). *myod* probe was prepared as described in E. Schnapp and colleagues (Schnapp et al., 2009); *mesp-a*, *her1*, *gadd45β1*, and *gadd45β2* plasmids were a kind gift from I. Dawid. *gsc, ntla*, and *shh* plasmids were a kind gift from P. Sordino. To synthesise the *ccdc80* probe, the same primers were used as for the RT-PCR.

For histological sections, stained embryos were re-fixed in 4% PFA, dehydrated and stored in methanol, wax embedded and sectioned (5 μm).

Images of embryos and sections were acquired using a microscope equipped with a digital camera with LAS Leica imaging software (Leica, Wetzlar, Germany). Images were processed using the Adobe Photoshop software and when necessary, different focal planes of the same 3D image have been taken separately and later merged in a single image.

Cyclopamine treatment

For the inhibition of the *Hh* pathways, embryos were exposed to 50 μM cyclopamine (Sigma-Aldrich, Inc, St. Louis, MO) from 50% epiboly stage up to fixation in PFA at 12–15 s stage. Cyclopamine was dissolved in embryo medium and 0.5% ethanol. Controls consisted of corresponding incubations in 0.5% ethanol in embryo medium (Barresi et al., 2000).

Results

Molecular cloning and in-silico analysis of the zebrafish *ccdc80*

In-silico search for the zebrafish *ccdc80* gene with the rat *Ccdc80 (Cl2)* virtual probe allowed the identification of exon homologous sequences in the genomic *Danio rerio* database (Zv9). The RT-PCR from the presumptive ATG to the stop codon, amplified a cDNA of 2604 bp in length. The sequence analysis of this product revealed only one ORF. The 5′ (189 bp) and 3′ (133 bp) UTR sequences were finally obtained by RACE. The 5′ ending of the gene did not show further alternative starting codons located in frame with the putative ORF. The final zebrafish *ccdc80* mRNA sequence (2926 bp) has been deposited in the Genbank database with the Accession Number EU269066 (GI:166831573).

ccdc80 cDNA codes for a protein of 867 amino acids in length. Zebrafish Ccdc80 is a highly basic protein (pI 9.68) and has a predicted molecular mass of 99.92 kDa. Sequence analysis shows a putative signal peptide and multiple nuclear localization signals. Moreover, it shows a putative endoplasmic reticulum retention signal at N-terminus (RARY), putative N-glycosylation sites and several mucin-type-O-glycosylation sites in a threonine-rich region from approximately amino acid 303–369 (Fig. 1A). Based on these results it is likely that the protein is post-translationally modified and can have both nuclear and cytoplasmatic localization. Moreover, along the

protein three clusters of sequences occurs with the following *consensus* sequences:

SLANFLASFAWKNRLWVISAPHAENWMYVQQMSAL NEQFCC, FFMVLVDKDLNVKQWYPYPMW and MEIMYDLI DQFQIRIQEMIIQKGFGMRCK; the alignment of the three domains (146, 151, and 149 amino acids in length, respectively) shows an overall identity of 26% and a similarity of 43% (Fig. 1B–C).

The entire zebrafish Ccdc80-encoded protein shares high sequence identity with human DRO1 (56% identity, 69% similarity), mouse Urb (55% identity, 68% similarity), rat Cl2 (54% identity, 68% similarity), and chicken L-EQUARIN (59% identity, 71% similarity) (Suppl. Fig. 1). A previous description and the alignment of the predicted zebrafish proteins Ccdc80 (Chr9:35,060,460–35,084,513), Ccdc80-like1 (ccdc80-l1, Chr6:16,322,724–16,342,517) and Ccdc80-like2 (ccdc80-l2, Chr21: 18,662,129–18,669,986) has been performed by Brusegan and colleagues (Brusegan et al., 2012).

Expression pattern of the zebrafish *ccdc80* gene in the adult fish and during embryonic development

Semi quantitative RT-PCR analysis on RNA extracted from organs of the adult fish (Fig. 2A) showed that *ccdc80* is expressed in all the analyzed tissues. In particular, it was expressed at the highest level in the heart, and at the lowest in the liver and intestine. Intermediate levels were evident in the brain, in the ovarian and surrounding adipose tissue, in the mesonephric duct, swim bladder, and pancreas.

Semi-quantitative temporal expression profile of *ccdc80* in zebrafish embryos at different developmental stages, showed the presence of the transcript from the early blastula up to 48 hours post fertilization (48 hpf) (Fig. 2B). In particular, *ccdc80* expression was decreased during gastrulation, presented a new peak of expression during somitogenesis, and maintained constant levels later in the development.

Spatial expression of *ccdc80* mRNA analyzed by WISH in pre-gastrulation (1–2, 4–8, 512–1000 cells) and gastrulation developmental stages (30%–70% epiboly) showed a diffuse pattern (Suppl. Fig. 2). From the 12-somite stage (12 s) to 48 hpf, *ccdc80* transcript was only detected in the entire notochord (Fig. 2C–H). Consistently with the semi quantitative RT-PCR analysis, transient expression appeared in the heart at 48 hpf (Fig. 2H–I).

ccdc80 is positively regulated by the *Hedgehog (Hh)* pathway

Considering the expression of *ccdc80* in the notochord, we decided to analyze its possible interaction with *Hedgehog (Hh)* pathway, that is one of the main pathways in the notochord that influences muscle differentiation (Echelard et al., 1993; Marti et al., 1995), and that is able to induce sclerotome and muscles in the absence of the notochord (Fan et al., 1995; Munsterberg et al., 1995). The blockage of *Hh* signaling in wild-type embryos treated from early cleavage stages with cyclopamine (Incardona et al., 1998; Barresi et al., 2000) (65%, N = 95; Figs. 3A–B and D), or in *syu* mutant embryos (Karlstrom et al., 1999) (70% N = 30, Fig. 3D) produced an inhibition of *ccdc80* expression in the notochord in comparison to controls (0%, N = 50; Fig. 3D). Conversely, the over-expression of the synthetic *shh* mRNA at

Fig. 1. Zebrafish Ccdc80 protein structure and motif analysis. (A) Image is displayed with the software PROSITE tool MyDomains - Image Creator. SP: Signal Peptide, CL: cleavage site, NLS: Nuclear Localization Signals, D1, D2, D3: domains, Thr: Threonin rich region. (B) Position of the three motifs (M1, M2, and M3) in the human DRO1, mouse Urb, rat Cl2, chicken EQUARIN-L, and zebrafish Ccdc80 homologous proteins. (C) Alignment of the zebrafish Ccdc80 D1, D2, D3 domains. Color legend: red, small, hydrophobic, and aromatic (-Y) residues; Blue, Acidic residues; Magenta, Basic residues; Green, Hydroxyl, Amine, Basic (-Q) residues. Consensus symbols: "*" identical residues in all sequences; ":" conserved substitutions; "." semi-conserved substitutions.

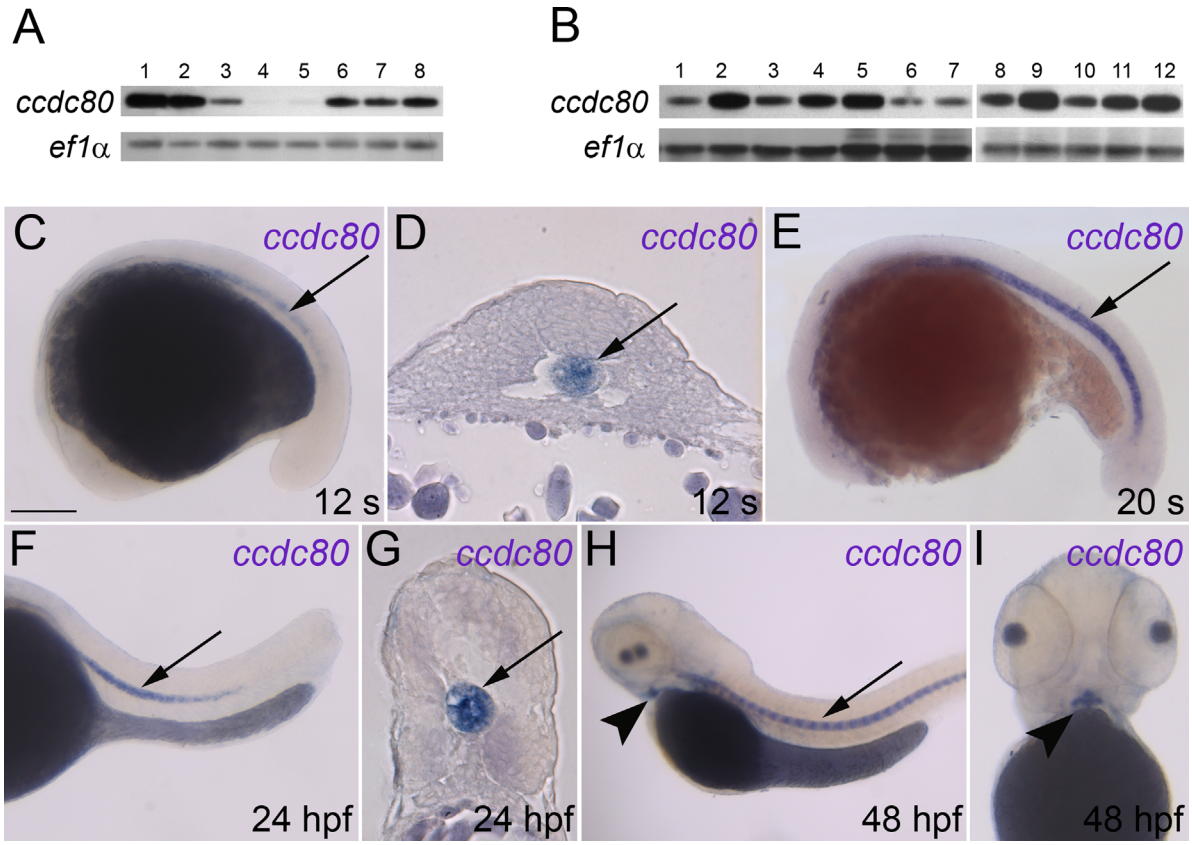

Fig. 2. Expression of *ccdc80* analyzed by semi quantitative RT-PCR and WISH. (A) Semi quantitative RT-PCR performed on different adult tissues (A) and embryonic stages (B); the expression of *ccdc80* and *ef1a* are shown. In (A) Lanes are: heart (lane 1), swim bladder (lane 2), pancreas (lane 3), liver (lane 4), intestine (lane 5), brain (lane 6), mesonephric duct (lane 7), ovary (lane 8). In (B) lanes are 1–2 cells (lane 1), 4–8 cells (lane 2), 128 cells (lane 3), 1000 cells (lane 4), 30% epiboly (lane 5), 50% epiboly (lane 6), 75% epiboly (lane 7), 1–3 somites (lane 8), 16 somites (lane 9), 22 somites (lane 10), 24 hpf (lane 11) 48 hpf (lane 12). (C–I) WISH performed on zebrafish embryos at several stages of development. (C–E) Starting from somitogenesis, *ccdc80* is expressed in the notochord (C and E, arrow), as better shown by the histological section of the trunk at 12 somites stage (arrow in D). The same hybridization signal persists also at 24 hpf (F and histological section G, arrow) and 48 hpf (H, arrow). At 48 hpf, *ccdc80* is also expressed in the heart (H and I, arrowhead). (C, E, F, H) Later views, dorsal is up. (D, G) Transversal sections of the trunk, dorsal is up. (I) Frontal view of the head, dorsal is up. Scale bar indicated 100 μm.

1-to-2 cell stage, induced *ccdc80* up-regulation in this structure (59%, N = 89; Fig. 3C, D). These results show a relationship between *ccdc80* and *Hh* pathway, in particular *ccdc80* appears to be an *Hh* downstream effector.

ccdc80 loss-of-function affects zebrafish somite formation

To determine the role of *ccdc80* during zebrafish development, we performed the knock-down of *ccdc80* by means of the injection of a specific antisense oligonucleotide morpholino designed against the start site (*ccdc80*-MO1, Gene Tools). In all the experiments, *ccdc80*-MO-injected embryos were compared to embryos at the same developmental stage, injected with the same amount of a non-specific control MO (ctrl-MO). At 15 s, the majority of *ccdc80*-MO1 injected embryos (75%; N = 150) displayed defects in the forming somites, such as the loss of their proper morphology and position, in comparison to control embryos (2%; N = 140, Figs. 4A–C). Although at 24 hpf the differentiated somites partially recovered their proper morphology and the embryos were normally moving, 64.5% of *ccdc80*-MO injected embryos (N = 124, Fig. 4E) showed curved tails compared to controls (1%, N = 90, Fig. 4D). For testing the in-vivo efficiency of *ccdc80*-MO1, 300 pg/embryo of the pCS2/GFP, containing the *ccdc80* target site of *ccdc80*-MO fused with the *gfp* ORF were co-injected with 12 ng of *ccdc80*-MO or ctrl-MO respectively (Fig. 4F–F'). The GFP signal was monitored under a fluorescent microscope at 20 s stage. The reduction of the GFP signal in the majority (98%; N = 81) of the embryos co-injected with *ccdc80*-MO1 and pCS2/GFP (Fig. 4G–G') indicated that *ccdc80*-MO1 efficiently binds to its target region in-vivo.

In parallel we performed loss-of-function experiments with a second morpholino (*ccdc80*-MO2, Gene Tools) designed in the 5'UTR, in a pre-ATG region. Injection with 12 ng/embryo of *ccdc80*-MO2 gave the same defects in somite formation as *ccdc80*-MO1, confirming the specificity of the loss-of-function phenotype (70%; N = 130, Fig. 4C). Moreover, the injection of a *ccdc80*-5'-mismatch-MO did not produce any phenotype. We were also able to partially rescue the phenotype co-injecting the *ccdc80*-MO1 with 300 pg/embryo of *ccdc80* full-length mRNA (30%; N = 230, Fig. 4C).

To address whether the observed somite malformations might be due to defects in gastrulation, we analyzed the expression pattern of critical markers of convergence and extension processes such as *goosecoid* (*gsc*) expressed in the shield area (Thisse et al., 1994) (Suppl. Fig. 3A–B), and *no tail a*

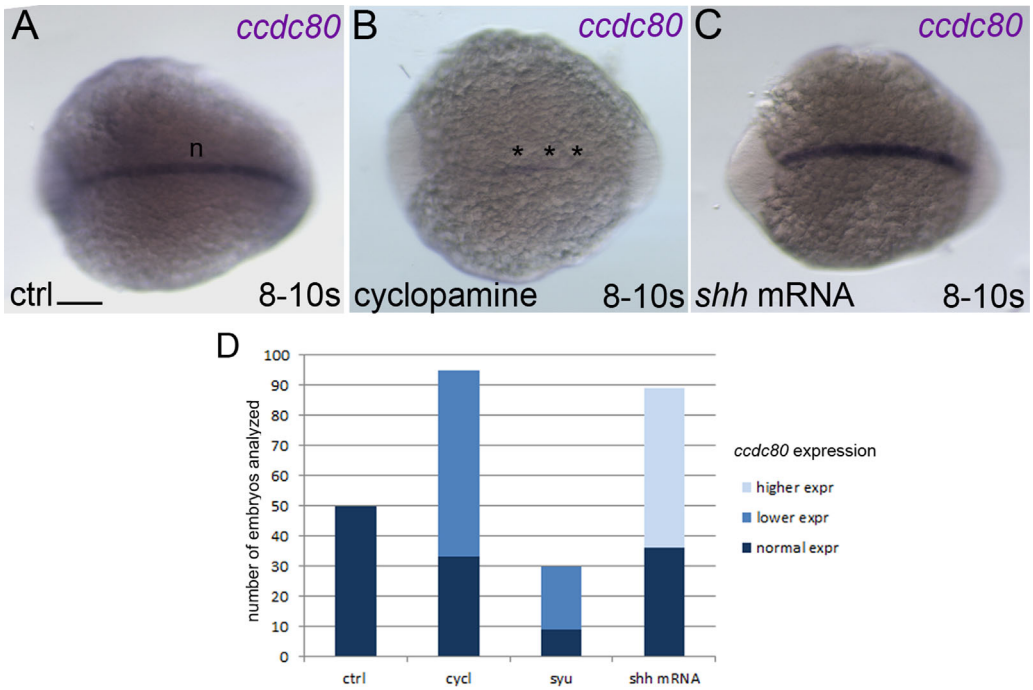

Fig. 3. shh regulates *ccdc80* expression. (A–C) In comparison with control embryos (A), cyclopamine treatment strongly down-regulated *ccdc80* expression in the notochord (B, asterisks), whereas injection of *shh* mRNA led to its up-regulation (C). (D) Different classes of embryos with an altered *Hh* signaling (ctrl, cyclopamine treated, *syu* mutants, *shh* mRNA injected), were analyzed for the expression levels of *ccdc80* in the notochord. (A–C) Dorsal views, rostral on the left. n: notochord. Scale bar indicated 100 μm.

(ntla) expressed in the notochord (Schulte-Merker et al., 1994) (Suppl. Fig. 3C–D). Although *ccdc80* is expressed in a diffuse pattern during gastrulation (Suppl. Fig. 2D) and in the whole notochord during segmentation (Fig. 3C–H), *ccdc80*-loss-of-function experiments did not affect the expression of these markers.

According to our previous demonstration of *ccdc80* regulation by *Hh* pathway, we decided to investigate whether the opposite effect also exists, by analyzing *shh* expression pattern in *ccdc80*-MO1 injected embryos. *shh* transcript was correctly expressed in all territories of *ccdc80*-loss-of-function embryos (Suppl. Fig. 3E–F), suggesting the lack of a feed-back-loop regulation effect.

ccdc80 loss-of-function perturbs the expression of markers of somitogenesis and myogenesis

To better characterize the molecular events underlying the somite defects observed in *ccdc80*-loss-of-funtion embryos, we examined the expression of genes implicated in somitogenesis and subsequent myogenesis. The first step in somite formation is the segmentation-clock that creates oscillations in gene expression in the PSM and allows somite primordium maturation. Several genes are expressed in the segmentation-clock period; among them critical markers are *her1* (Holley et al., 2000) and *mesp-a* (Durbin et al., 2000; Sawada et al., 2000). In *ccdc80*-loss-of-function embryos, *her1* (Fig. 5A–B, 80%, N = 60) and *mesp-a* (Fig. 5C–D, 70%, N = 60) expressions were affected, showing irregular asymmetric patterns and/or broader domains rather than the defined territories present in control embryos at the same developmental stages. Furthermore, in *ccdc80*-MO injected embryos, the myogenic marker *myod* was present but disorganized in the paraxial mesoderm, reflecting upstream defects in somitogenesis (Fig. 5E–F, 81% N = 60).

The cell cycle controllers *gadd45β1* and *gadd45β2* genes have also been reported to control segmentation process and somite development (Kawahara et al., 2005). For this reason, we decided to investigate their expression in control and *ccdc80*-loss-of-function embryos. *gadd45β2* expression was almost completely abolished in paraxial mesoderm in most of the *ccdc80*-MO1 injected embryos (90%; N = 88, Fig. 5G–H') while it was invariant in the PSM and Lateral Plate Mesoderm (LPM) domains (Fig. 5G–H'). On the contrary, *gadd45β1* expression was not significantly changed in *ccdc80*-loss-of-function embryos (data not shown). We were also able to rescue the *ccdc80*-loss-of-function phenotype: the co-injection with 200 pg/embryo of *gadd45β2* full-length-mRNA partially rescued both the *ccdc80*-MO1 induced alterations of somite structures and the *myod* expression at 8–10 s stage (30%; N = 55, Suppl. Fig. 4A–C'). Interestingly, *ccdc80* mRNA overexpression caused the opposite effect: an enlargement of *gadd45β2* paraxial mesoderm expression domain in the majority (60%; N = 70, Fig. 5I–I') of injected embryos, and an ectopic *gadd45β2* expression in non-mesodermal territories such as neural tube (Fig. 5I–I'). Moreover, we observed a decreased or absent *gadd45β2* expression in LPM territories, while PSM was unaltered (Fig. 5I–I').

The modulation of the *Hedgehog* pathway affects *gadd45β2* expression

To examine whether the interaction between *Hh* pathway and *ccdc80* in the notochord has an effect on the regulation of *gadd45β2* in the somites, we analyzed *gadd45β2* expression in

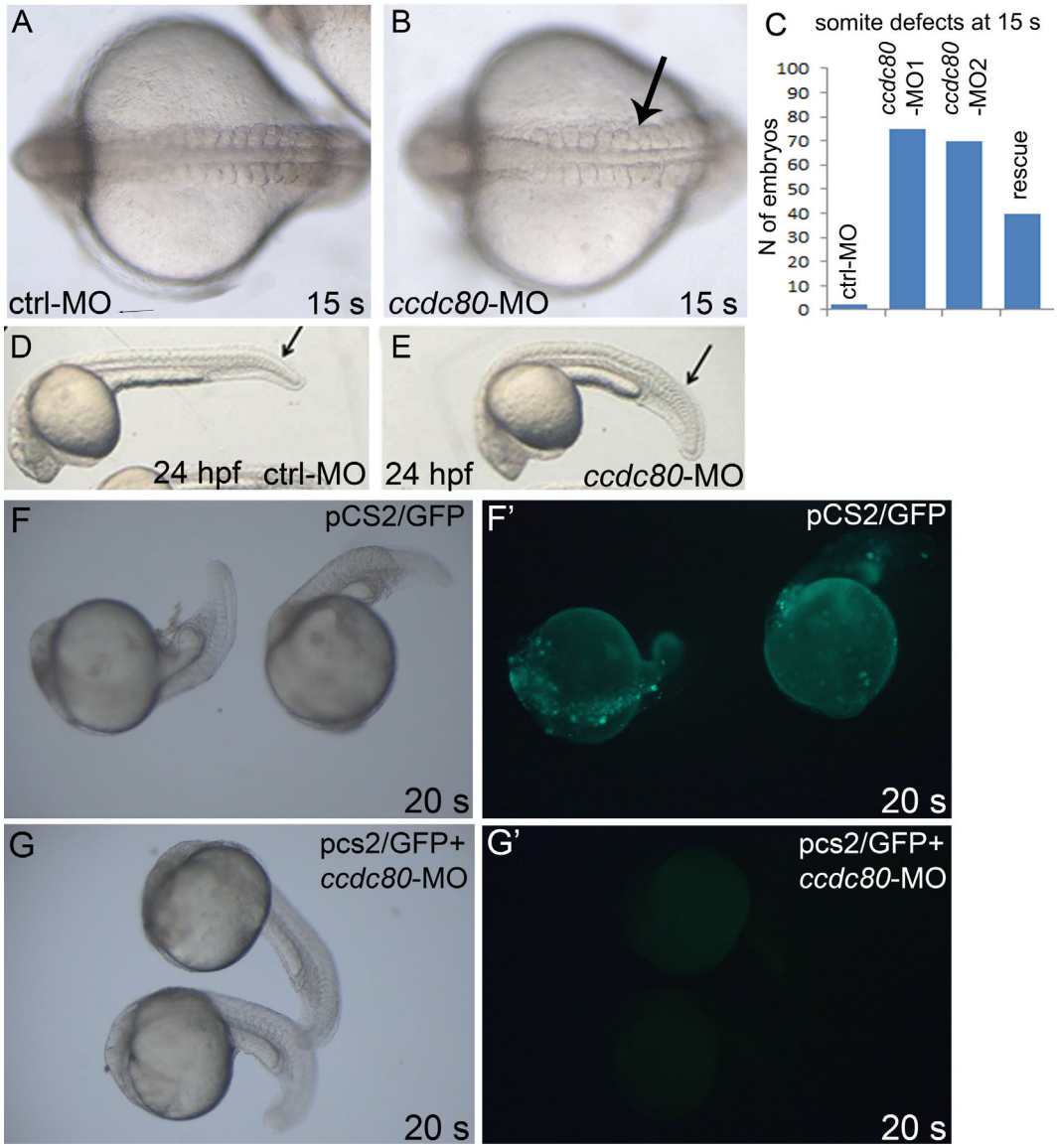

Fig. 4. Loss-of-*ccdc80* function affect somitogenesis in-vivo. (A–B) *ccdc80*-MO injected embryos (B) displayed enlarged and disorganized somites in comparison to control embryos (A). Smaller, supernumerary somites are visible in *ccdc80*-MO injected embryos (arrow in B). (C) Number of embryos showing somite defects following *ccdc80*-mis-regulation (*ccdc80*-MO1, *ccdc80*-MO2, and rescue), in comparison to controls at 15 s. (D–E) *ccdc80*-MO injected embryos (arrow, E) displayed a curved tail in comparison to control embryos at 24 hpf (arrow, D). (F, G') The pCS2+ construct containing the sequence recognized by the *ccdc80*-MO fused with the *gfp* open reading frame is used for injection experiments with ctrl-MO or with the *ccdc80*-MO. (F–F') Embryos at 20 s stage: GFP-positive cells in the trunk and in the yolk epithelium following co-injection of the sensor and the control-MO. (G–G') The absence of GFP when the sensor is co-injected with *ccdc80*-MO confirms the efficiency of morpholino to target the *ccdc80*-ATG region. In F and G embryos are visualized under normal light, in *F'* and *G'* under fluorescent light. (A–B) Dorsal views, rostral to the left. (C–D) Lateral views, rostral to the left. Scale bars indicated 100 μm.

embryos inhibited in the *Hh* pathway. Interestingly, the inhibition of the *Hh* pathway with cyclopamine treatment, resulted in a depletion of *gadd45β2* expression in the paraxial mesoderm (Fig. 6A–B'), as previously shown in *ccdc80*-loss-of-function embryos (Fig. 5H–H'). Conversely, the expression of *gadd45β2* in the PSM was unaltered.

Discussion

Somitogenesis is a developmental process by which the vertebrate trunk is divided into a series of segments termed somites. In zebrafish, somites form in an anterior to posterior progression from the unsegmented PSM. This process requires a segmentation clock that comprises oscillations in gene expression in individual cells within the PSM. The coordination of these mechanisms is guaranteed by several molecular pathways; among them a pivotal role is played by the *Hedgehog* signaling secreted by the notochord (Lewis et al., 1999; Wolff et al., 2003). Mutants lacking the notochord often present somitogenesis defects (reviewed by Stickney et al., 2000) and modulation of Shh protein affects the proper formation of somites and muscles (reviewed by Ingham and Kim, 2005). In zebrafish *ccdc80* is expressed during gastrulation and, by the time the somites are formed and specified, in the notochord. Its

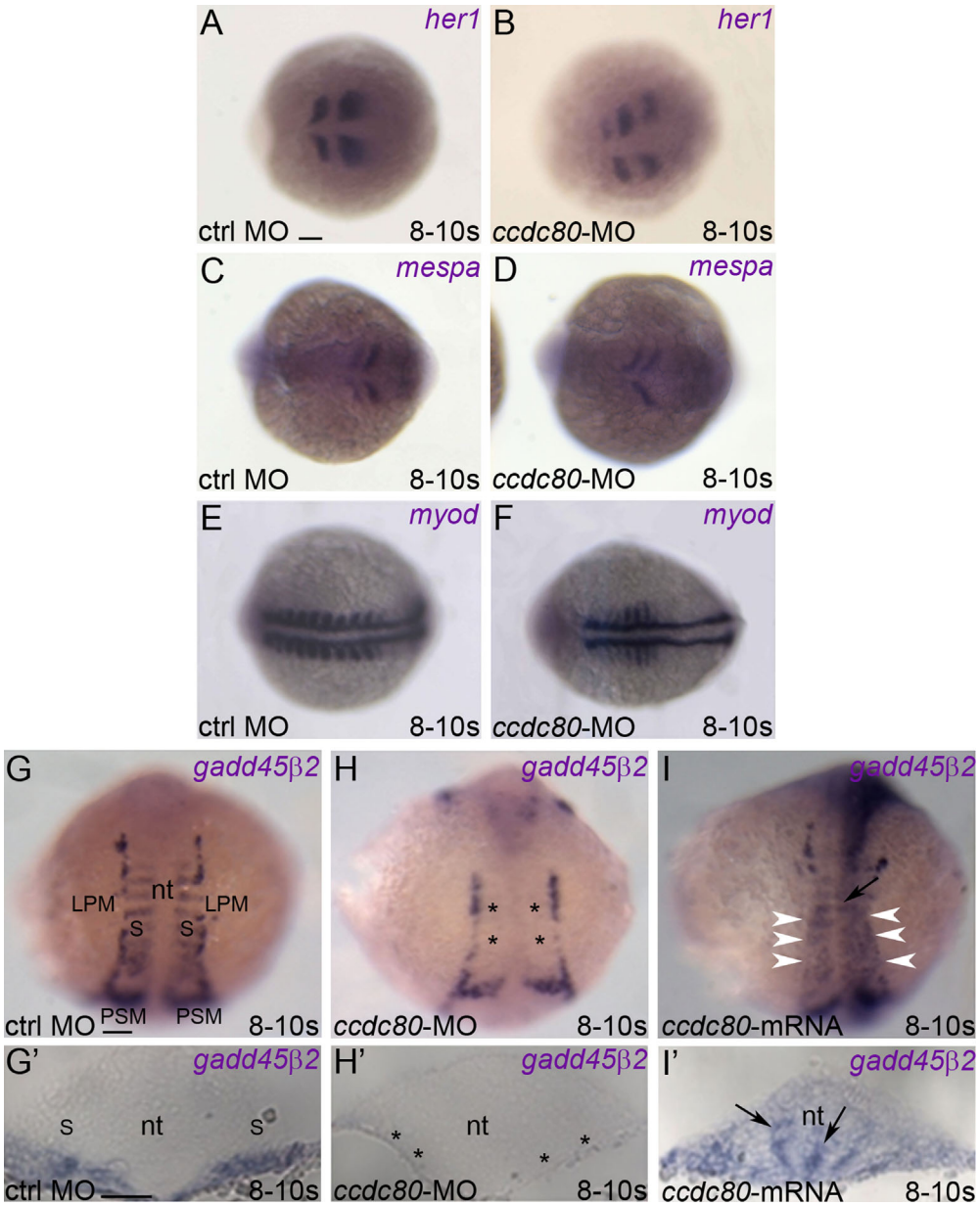

Figure 5. ***ccdc80*** **loss-of-function perturbs the expression of markers of somitogenesis and myogenesis. (A–D) Expression of the molecular clock genes** ***her1*** **(A–B) and** ***mesp-a*** **(C–D) were altered in** ***ccdc80*****-loss-of-function embryos in comparison to controls at the same developmental stage. The myogenic marker** ***myod*** **(E–F) was disorganized in** ***ccdc80*****-injected embryos, reflecting previous defects in somitogenesis.** ***gadd45β2*** **expression resulted almost completely abolished in the paraxial mesoderm of** ***ccdc80*****-MO injected embryos (H–H', asterisks) in comparison to controls (G–G').** ***ccdc80*** **mRNA over-expression caused the opposite effect with a broader** ***gadd45β2*** **expression domain also in ectopic position in non-mesodermal territories (*I-I'*, black arrows) and a decreased signal in the LPM (I–I', white arrowheads). (A–F) Dorsal views, rostral to the left. (G, H, I) Dorsal view, head is up. (G', H', I') Transversal sections of the trunk, dorsal is up. LPM: lateral plate mesoderm, nt: neural tube, S: somites. Scale bars indicated 100 μm.**

function is not fundamental for notochord development, as injection of *ccdc80* morpholino did not impair the formation of medial structures. Moreover, despite its zygotic expression, *ccdc80* is not required during gastrulation as shown by the proper expression of the organizer gene *gsc* and the mesodermal marker *ntla*. By converse, *ccdc80*-MO injected embryos displayed severe defects during somitogenesis, suggesting that *ccdc80* might be secreted from the notochord. Indeed, Ccdc80 protein shows a signal peptide besides nuclear localization signals that may allow its secretion and internalization respectively. Investigation on Ccdc80 cellular localization in zebrafish is still needed, whereas several studies already indicated human, rat, mouse and chicken homologous as secreted proteins (Mu et al., 2003; Visconti et al., 2003; Liu et al., 2004; Bommer et al., 2005; Ferragud et al., 2011). Analysis of the expression patterns of different markers of somitogenesis in *ccdc80*-loss-of-function embryos confirmed that somites lost their proper pattern. In particular, synchronization of molecular clock oscillations seemed to be lost, as the expression of *her1* resulted asymmetric and disorganized. Consequently, the expression pattern of the down-stream markers *mesp-a* and *myod* resulted altered. The phenotype

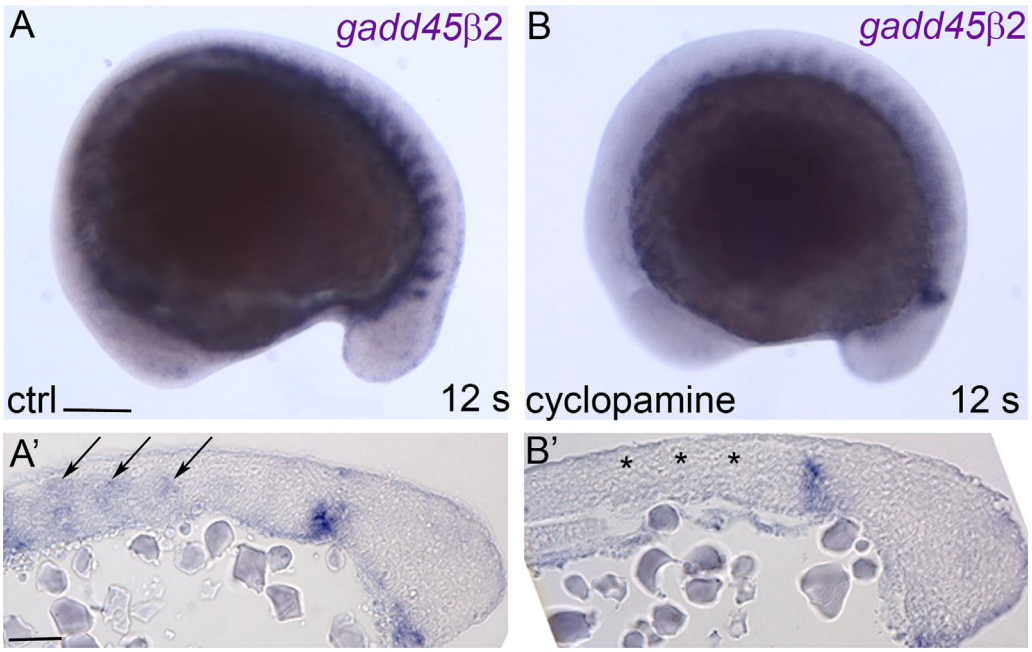

Figure 6. gadd45β2 expression is modulated by the Hedgehog pathway. (A–B') The expression of *gadd45β2* is diminished in the paraxial mesoderm of embryos treated with the *Hh* pathway inhibitor cyclopamine (B–B') in comparison to control embryos at 12 s (A–A'). (A, B) Lateral views, rostral to the left. (A', B') Flat mount of the trunk and tail regions showing absence of *gadd45β2* expression in the somites of cyclopamine treated embryos (asterisks) in comparison to controls (arrows). Rostral to the left. Scale bars indicated 100 μm.

displayed by *ccdc80*-MO injected embryos is recovered later in development and motility returns close to normality at 24 hpf. Hence, *ccdc80* function may be relevant, but not necessary, for somite formation and not determinant for subsequent somite function maintenance; this is consistent with the abundance of pathways regulating this process and the complexity of positive and negative feedback loops involved (Mara and Holley, 2007). In support of the hypothesis of Ccdc80 secretion by the notochord, we demonstrated its interaction with the *Hedgehog* pathway. Expression of *ccdc80* resulted directly regulated by the levels of Shh protein, and the opposite phenomenon was never observed. These findings strongly indicate *ccdc80* as a down-stream effector of *shh*: its expression in the notochord is promoted by *shh*, and the protein is likely released in the surrounding tissues, contributing to somitogenesis. Thereafter, we identified *gadd45β2* as a potential *ccdc80*-target in this process. *gadd45β* genes in zebrafish are expressed in the PSM and in the somites, they are regulated by the segmentation clock and are fine tuned in somite boundary formation (Kawahara et al., 2005). In mammals and in zebrafish, GADD45 proteins are involved in cell-cycle control, interacting with MTK1/MEKK4 factors that are required for somite segmentation (Mita et al., 2002; Kawahara et al., 2005). In addition, Gadd45 family members were found to be involved in genome demethylation in zebrafish (Rai et al., 2008), suggesting an important role for these factors in muscle development. Indeed, the transcription activity of *MYOD* and *MEF2* is epigenetically regulated by histone methyltransferases (HMTs), histone deacetylases (HDACs) and histone acetyltransferases (HATs) (Caretti et al., 2004; Sartorelli and Caretti, 2005; Mal, 2006) and changes in the methylation status of the human *MYOD* CpG island might lead to oncogenic transformations (Rideout et al., 1994).

Interestingly, the knock-down of *gadd45β2* (Kawahara et al., 2005) causes the same defects as *ccdc80* knock-down and the injection of *gadd45β2* mRNA partially rescued the *ccdc80*-loss-of-function phenotype, indicating its down-stream position in the *ccdc80* pathway.

This study demonstrates for the first time the expression of *ccdc80* in notochord during zebrafish development and its role in somite formation and patterning. Moreover, our data identify *ccdc80* as a down-stream effector of the notochord *Hedgehog* signaling and an up-stream regulator of *gadd45β2* in somite formation.

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Author Contributions

Conceived and designed the experiments: AP, IDN, CB, SC, FS, FC. Performed the experiments: AP, IDN, CB, SC, AF, CDL, RC. Analyzed the data: AP, IDN, CB, SC, FS, FC. Contributed reagents/materials/analysis tools: FS, FC. Wrote the paper: AP, CB, IDN. Supervised the research project: FC, AP, FS. Supervised paper drafting: FC, AP, GB, AG, EV, FS.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Supplementary material

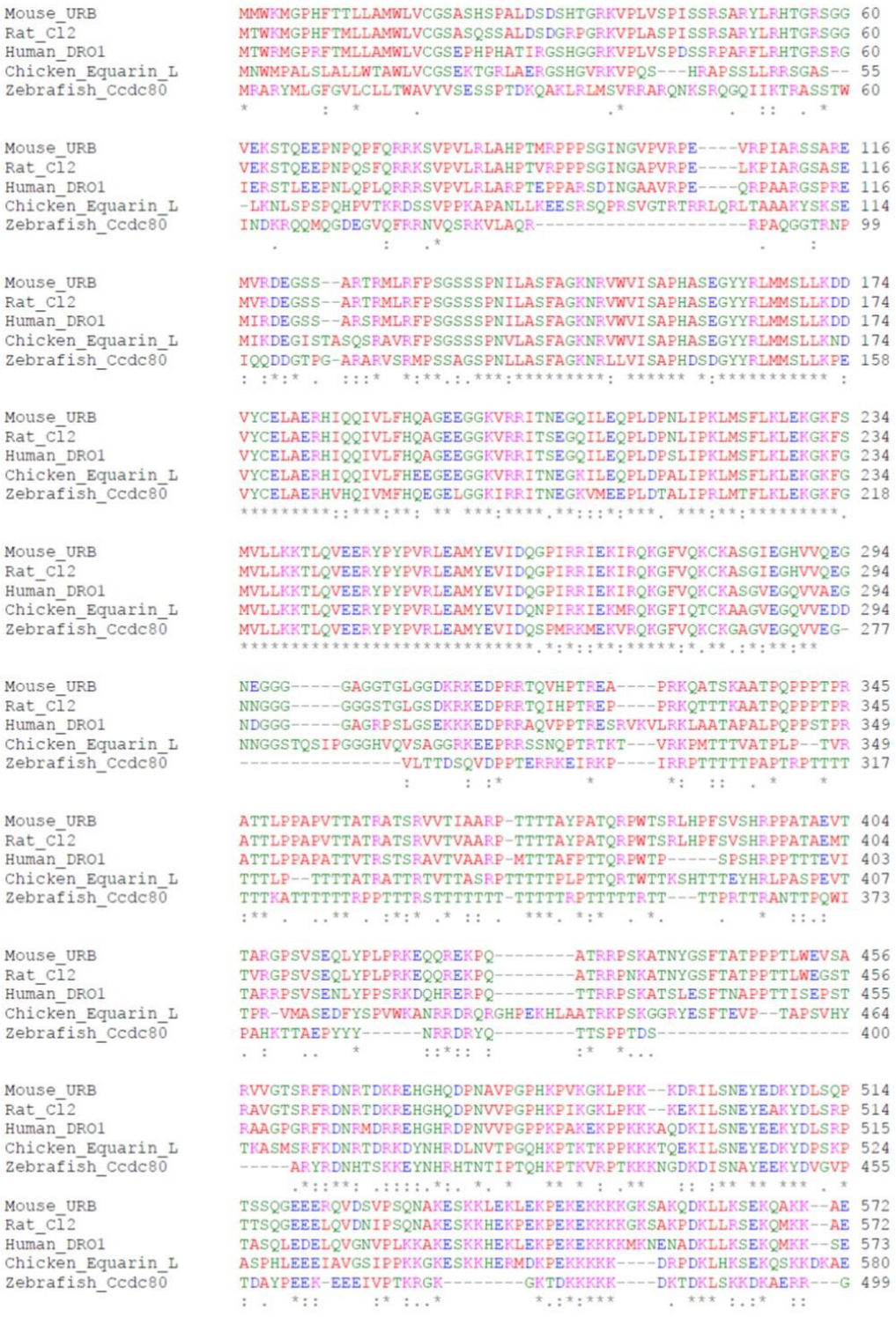
Suppl. Figure 1: ClustalW2 alignment of CCDC8 homologous. Color legend: red, small, hydrophobic, and aromatic (-Y) residues; Blue, Acidic residues; Magenta, Basic residues; Green, Hydroxyl, Amine, Basic (-Q) residues. Consensus symbols: "*" identical residues in all sequences; ":" conserved substitutions; "." semi-conserved substitutions.

Suppl. Figure 2: *ccdc80* expression in early developmental stages. Diffuse *ccdc80* expression at 1 cell *(A)*, 4 cells *(B)*, 1000 cells *(C)* and 30% epiboly *(D).* Scale bar indicated 250 μm.

Suppl. Figure 3: critical markers for gastrulation and convergence and axis formation are not affected in *ccdc80*-MO injected embryos. *(A-B) gsc*, marker of epiboly, resulted correctly expressed at shield stage in *ccdc80*-MO injected embryos. *(C-D)* Also the expression pattern of *ntla*, marker of the notochord, resulted unaltered in all embryos during somitogenesis. *(E-F) shh* expression was not perturbed by the *ccdc80*-MO injected embryos in comparison to controls. *(A-B)* Lateral views, dorsal to the right. *(C-F)* Dorsal views, rostral to the left. Scale bar indicated 100 μm.

Suppl. Figure 4: Rescue the *ccdc80*-loss-of-function phenotype with the co-injection of *gadd45β2* full-length-mRNA. *myod* expression in controls *(A-A')*, *ccdc80*-MO *(B-B')* and *ccdc80*-MO+*gadd45β2*mRNA-injected embryos at 8-10 s stage. *(A-B-C)* Lateral views, rostral to the left. *(A'-B'-C')* Dorsal views, rostral to the left. Scale bar indicated 100 μm.

Suppl. Figure 1



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Human_DRO1             KKSKQEKEKSKK-KKGGKTEQDGYQKPTNKHFTQSPKK-SVADLLGSFEGKRRLLLITAP 631
Chicken_Equarin_L      KKSKQDKDRSKKNKKGSRTENEDFPKPNKKPFLQPPRK-SVANLLDYFEGKRRLILITTP 639
Zebrafish_Ccdc80       KDGKGGKKNGKK--VPKILEKEDYQKPTKRPPPPPPPPKGTLATFLDYFESRRRLILITSP 557
                       *. *  *.. **       *::.  **. :    .* * ::* :*. **.:***:***:*

Mouse_URB              KAENNMYVQQRDEYLESFCKMATRRISVVTIFGPVNNSSMKIDHFQLDNEKPMRVVDDDD 690
Rat_C12                KAENNMYVQQRDEYLESFCKIATRRISVVTIFGPVNNSSMKIDHFQLDNEKPMRVVDDED 690
Human_DRO1             KAENNMYVQQRDEYLESFCKMATRKISVITIFGPVNNSTMKIDHFQLDNEKPMRVVDDED 691
Chicken_Equarin_L      KADNTMYVQQRDEYLESFCKMATRKISVITIFGTMNNSSMKIDHFQLDNEKPMKVIEDED 699
Zebrafish_Ccdc80       TEENSMYIQQRDEYLEHVCEMAIRKVTIITIFGTFRNSTMKIDHYQLEKDKPMKGLRQED 617
                       . :*.**:******** .*::* *::::::****...**:*****:**:::***: : ::*

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Rat_C12                LVDQHLISELRKEYGMTYNDFFMVLTDVDLRVKQYYEVPIAMKSVFDLIDTFQSRIKDME 750
Human_DRO1             LVDQRLISELRKEYGMTYNDFFMVLTDVDLRVKQYYEVPITMKSVFDLIDTFQSRIKDME 751
Chicken_Equarin_L      LVDQQLISELRKEYGMTYNDFFMVLTDTDMKVKQYYEVPIAMKSVFDLIDTFQSRIKDME 759
Zebrafish_Ccdc80       LENQDLIMELRKEYGMTYNDFYVVLTDLDMKAKQYYEVPIAMKAVFDYIDTFSSRIREME 677
                       * :* ** **********:**::**** *::.********:**:*** ****.***::**

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Rat_C12                KQKKEGITCK-EDKRQSLENFLSRFRWRRRLLVISAPNDEDWAYSQQLSALNGQACNFGL 809
Human_DRO1             KQKKEGIVCK-EDKKQSLENFLSRFRWRRRLLVISAPNDEDWAYSQQLSALSGQACNFGL 810
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Zebrafish_Ccdc80       QQKRDGVTCKKEDKPRSLENFLSRFRWRRRLFVISAPNDEEWAYQQQLYALTSQACNLGL 737
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Human_DRO1             RHITILKLLGVG-EEVGGVLELFPINGSSVVEREDVPAHLVKDIRNYFQVSPEYFSMLLV 869
Chicken_Equarin_L      RHITILKLLGVG-EDIGGVLELYPINGSATVDREDIPANLVKDIRNYFQISPEYFSMLLV 877
Zebrafish_Ccdc80       RHVSVLKLVGTDLLDMGGVLELYPINGSATVEREGISATLVRDIRNYFQISPEYFSMLLV 797
                       **:::***:*..  ::**:***:*****: *:**.:.* **:*******:**********

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Rat_C12                GKDGNVKSWYPSPMWSMVIVYDLIDSMQLRRQEMAIQQSLGMRCPEDEYAGYGYHSYHQG 928
Human_DRO1             GKDGNVKSWYPSPMWSMVIVYDLIDSMQLRRQEMAIQQSLGMRCPEDEYAGYGYHSYHQG 929
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Human_DRO1             YQDGYQDDYRHHESYHHGYPY 950
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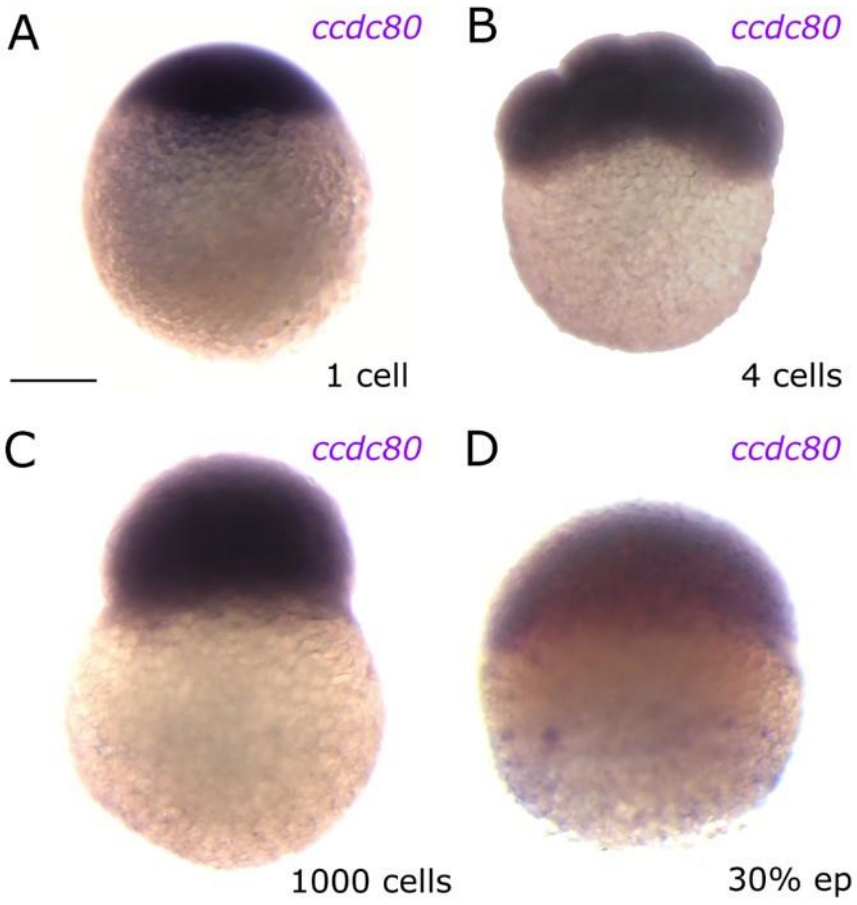
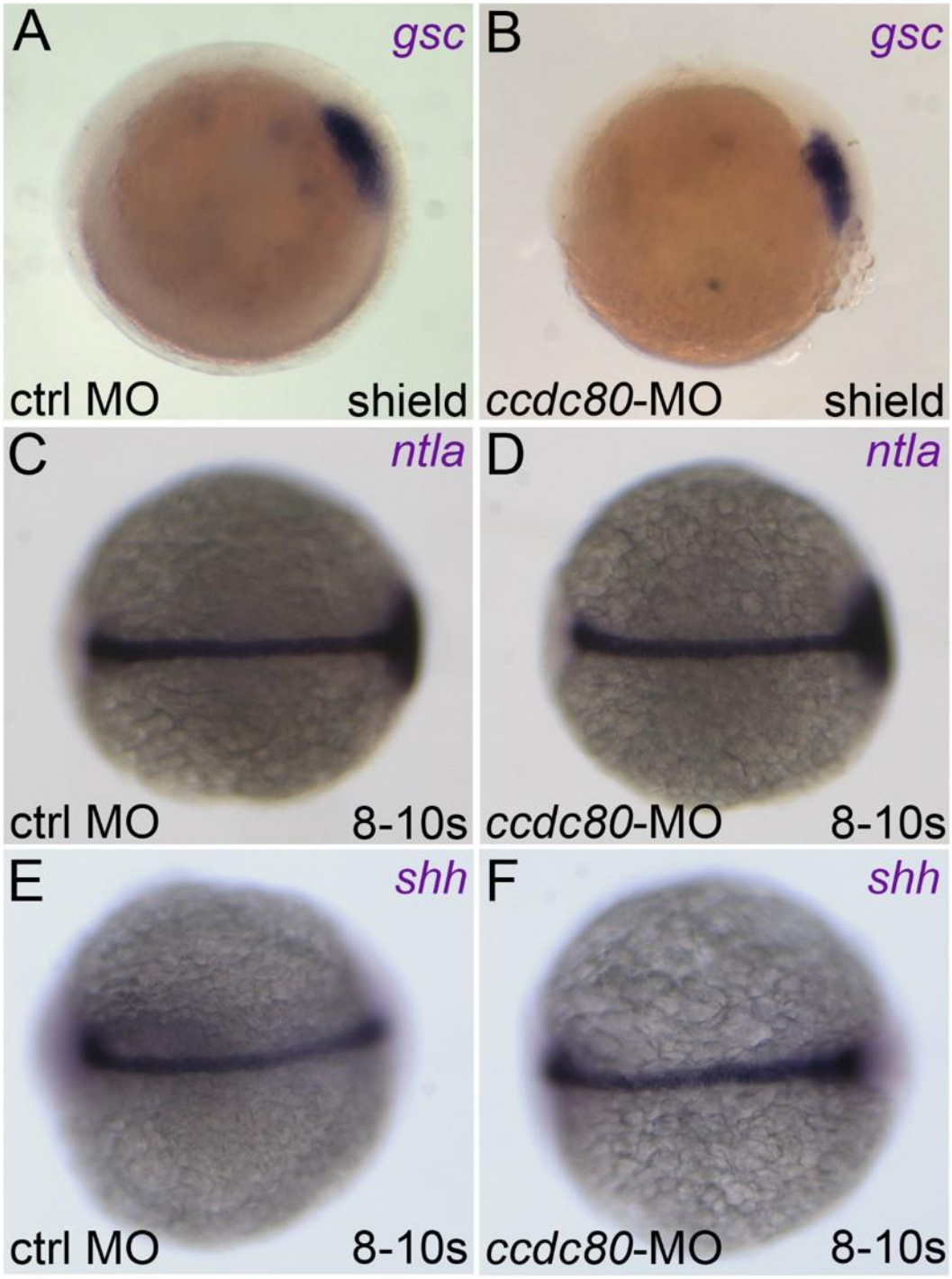
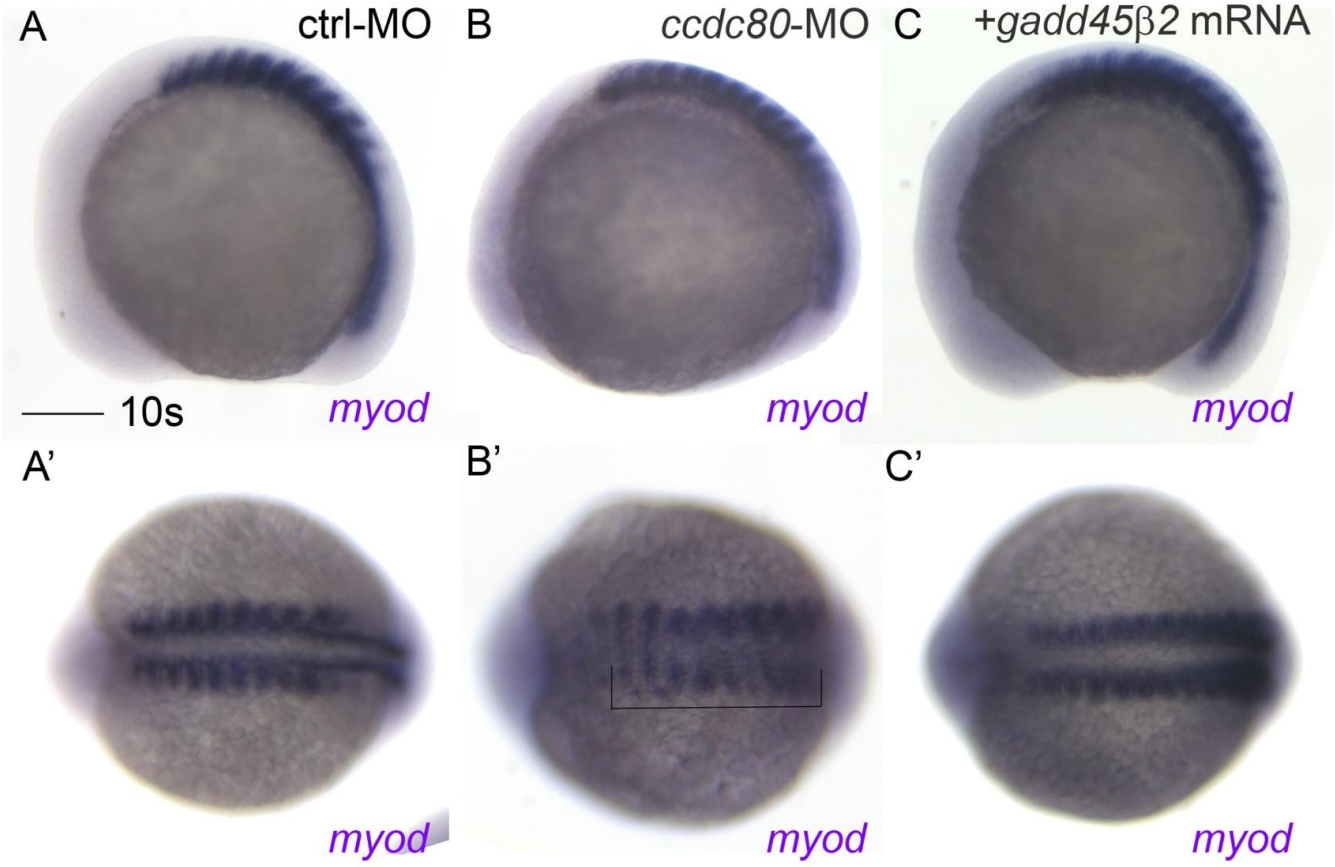
Suppl. Figure 2



Suppl. Figure 3



Suppl. Figure 4



Zebrafish embryo developmental stages