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Stat3-dependent mitochondrial DNA transcription drives stem cells proliferation in zebrafish

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ABSTRACT

The Signal Transducer and Activator of Transcription 3 (STAT3) is a transcription factor known to be involved in a plethora of physiological processes such as development, differentiation, immunity and metabolism, thus leading to transcription of anti-apoptotic, pro-proliferative and immune response target genes. Importantly, STAT3 pathway was also found aberrantly active in many human pathological conditions including 70% of solid and haematological tumours.

With the aim to investigate the putative role of Stat3 *in vivo* in physiological and pathological conditions, we generated and validated a transgenic line reporting the spatial and temporal expression pattern of canonical Stat3 pathway in zebrafish. It expresses a stabilized form of GFP fluorescent protein under the control of 7 multimerized STAT3 responsive elements from the promoter of the human STAT3 target gene *CRP*. The characterization of the reporter fluorescent pattern revealed that Stat3 activity is inherited maternally from the oocyte, and that the pathway is active during early developmental stages, in particular in the developing nervous system (telencephalon, optic tectum and hindbrain) from 20 hpf and in the intestine from 4 dpf until adulthood. In these tissues, Stat3 pathway is active in a restricted population of proliferating cells, in which, the chemical inhibition of Stat3 activity results in a significant reduction of the mitotic rate, suggesting that cell division is Stat3-dependent. In particular, in the intestinal epithelium of zebrafish larvae, Stat3 chemical ablation causes the depletion of intestinal folds formation, assessing Stat3 to be necessary for normal intestinal development. The generation of a novel *stat3-KO* mutant fish by Crispr-Cas9 mutagenesis confirmed the absolute requirement of Stat3 transcription factor for the formation of physiologic intestinal mucosa. In the adult zebrafish, Stat3 activity in the intestine is limited to the Crypt Base Columnar cells, a population of adult stem cells that is actively proliferating in order to sustain intestinal cells turnover. Moreover, in the zebrafish *apc*- genetic model for human colorectal cancer, Stat3 reporter activity is much increased in the intestinal adenomatous polyps, where it possibly marks the tumour initiating cancer stem cells, thus suggesting Stat3 to be a marker of stemness in the zebrafish.

According to some recent data collected *in vitro*, a limited pool of Stat3 localize to mitochondria, where it acts as a mitochondrial transcription factor inducing proliferation and maintaining pluripotency in ESC. In this work we provide *in vivo* evidences that mitoStat3 positively regulates embryonic proliferation through expression of mitochondrial genes, and

that in zebrafish, mitoStat3 transcriptional activity depends on both Y705 and S727 phosphorylation.

In its entirety, our work supports the idea that Stat3 regulates stem cells proliferation through mitochondrial genes expression in zebrafish.

SUMMARY

Il fattore trascrizionale STAT3 (Signal Transducer and Activator of Transcription 3), è noto per essere coinvolto in una varietà di processi fisiologici tra i quali lo sviluppo, il differenziamento cellulare, l'immunità e il metabolismo, attraverso la regolazione dell'espressione di geni target anti-apoptotici, pro-proliferativi e coinvolti nei meccanismi di risposta immunitaria. Inoltre, è stata osservata l'attivazione ectopica della via di segnale STAT3 in numerose patologie umane che includono il 70% dei tumori sia solidi che ematologici.

Con lo scopo di studiare *in vivo* il ruolo di Stat3 sia in condizioni fisiologiche che nel processo di patogenesi, è stata generata una linea transgenica biosensore della via canonica di segnale Stat3, in grado di riportarne l'attivazione spaziale e temporale nell'organismo modello zebrafish.

Essa esprime la proteina fluorescente verde EGFP sotto il controllo di 7 elementi ripetuti in tandem contenenti le sequenze responsive a Stat3; tali elementi, sono stati ottenuti dal promotore del gene *CRP* di uomo, un noto bersaglio dell'attività trascrizionale di Stat3.

La caratterizzazione del segnale fluorescente del pesce reporter ha dimostrato che la proteina Stat3 manifesta effetto materno, essendo ereditata dallo zigote a partire dall'oocita della madre; inoltre, la via di segnale è attiva durante le prime fasi dello sviluppo di zebrafish, in particolare nel sistema nervoso (telencefalo, tetto ottico e cervello posteriore) dalle 20 ore dopo la fecondazione, e nell'intestino a partire dai 4 giorni fino all'età adulta. In entrambi questi tessuti il segnale reporter marca cellule proliferanti, che a seguito della somministrazione di inibitori chimici della via di segnale Stat3 riducono significativamente la loro attività mitotica, suggerendo che il processo di divisione cellulare dipende da Stat3 durante lo sviluppo di zebrafish. In particolare nell'epitelio intestinale delle larve, l'inibizione chimica della via di segnale Stat3 causa difetti nella formazione dei villi intestinali, rivelando che Stat3 è necessario per il corretto sviluppo dell'epitelio intestinale in zebrafish. Tale risultato è stato avvalorato a seguito della creazione di un mutante KO per il gene *stat3,* il quale ha confermato l'assoluta necessità della proteina Stat3 per la formazione della normale mucosa intestinale. Nell'intestino di zebrafish adulti l'attività della via di segnale Stat3 è limitata ad una ristretta popolazione di cellule staminali adulte localizzate alla base della cripta, che proliferano attivamente per garantire il ricambio cellulare tipico di questo tessuto.

Inoltre, nello zebrafish modello per il tumore colon-rettale umano, caratterizzato da una mutazione al gene *apc,* l'attività della via di segnale Stat3 riportata dal pesce reporter è

significativamente maggiore nei polipi adenomatosi, dove verosimilmente è attiva nelle cellule staminali tumorali iniziatrici del cancro; tali risultati suggeriscono per Stat3 un ruolo come marcatore di staminalità in zebrafish.

Secondo alcune recenti pubblicazioni basate su dati ottenuti *in vitro,* una quantità limitata di proteina STAT3 è stata localizzata nel mitocondrio, dove agisce come fattore trascrizionale mitocondriale, inducendo la proliferazione e mantenendo la pluripotenza delle cellule staminali embrionali di topo. In questo lavoro vengono presentati dati ottenuti *in vivo*, a dimostrazione che mitoStat3 regola positivamente il processo proliferativo attraverso l'espressione di geni mitocondriali; si è inoltre dimostrato che l'attività trascrizionale di mitoStat3 dipende dalla fosforilazione ad entrambi gli amminoacidi Y705 e S727.

Nel suo complesso il nostro lavoro dimostra che il fattore trascrizionale Stat3 è in grado di regolare la proliferazione nelle cellule staminali attraverso l'espressione di geni mitocondriali in zebrafish.

1 INTRODUCTION

1.1 STAT3 and the JAK/STAT Signaling Pathways

The Janus kinase/Signal Transducers and Activators of Transcription (JAK/STAT) pathway is one of a handful of pleiotropic cascades able to transduce a multitude of signals for development and homeostasis in animals, from humans to flies (Rawlings et al., 2004).

First discovered during the studies on the specificity of IFN receptors, the Stats are a family of latent cytoplasmic proteins activated to participate in gene control when cells encounter various extracellular polypeptides.

To date, seven members of the STAT family of proteins have been identified in mammals: STAT1, 2, 3, 4, 5a, 5b, and 6. All family members share six distinct structural domains, including the N-terminal, coiled-coil, DNA-binding, linker, Src homology 2 (SH2), and the transactivation domains, that contain a critical tyrosine located around residue 700 that is obligatory for functional activation (Fig.1.1).

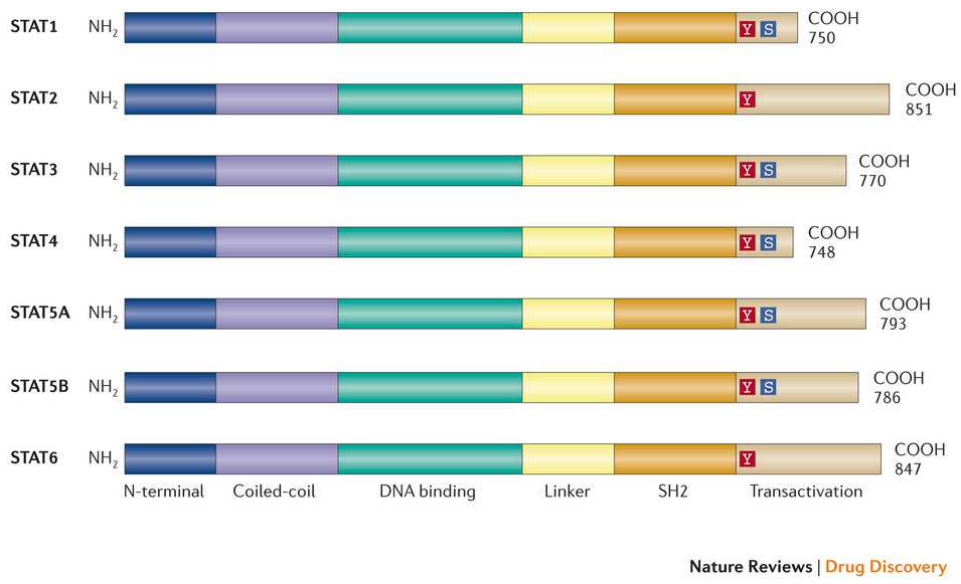

Figure 1.1 A schematic representation of STAT proteins functional domains. The seven members of the Stat family of transcription factors share 6 functional domains in humans. From Miklossy et al., 2013.

Different cytokines have the propensity to activate a particular STAT; however, interactive promiscuity between cytokines and any given STAT operates to various degrees (O'Shea et al., 2015).

Once a ligand interacts with the receptor, STATs are activated through phosphorylation of the key tyrosine residue, present in the STAT transactivation domain, by growth factor receptors, Janus kinases (JAKs), SRC family kinases and other tyrosine kinases. This leads to a cascade of events including STAT–STAT dimerization through a reciprocal phospho-tyrosine (pTyr)-SH2 domain interaction, nuclear translocation, DNA binding and the transcriptional induction of target genes in the nucleus. Physiological negative regulators, such as suppressors of cytokine signaling (SOCS) and protein tyrosine phosphatases (PTPs), ultimately downregulate the active STAT signaling (Miklossy et al., 2013).

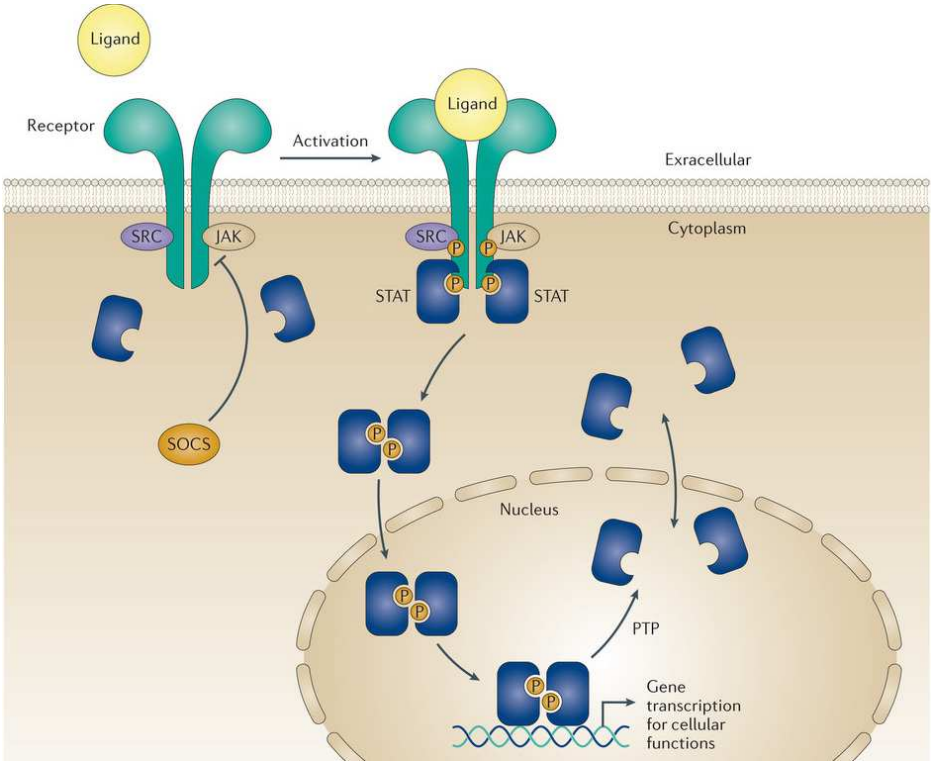

Figure 1.2 Stat signaling pathway. Activation of the Stat pathway occurs when a ligand binds to the receptor, inducing receptor phosphorylation via receptor associated kinases or via recruitment of cytoplasmic kinases. Cytoplasmic Stats are recruited by the phospho-motifs of the receptor and are their selves phosphorylated by tyrosine kinases. P-Stat monomers dimerize and enter the nucleus where they bind to specific Stat responsive elements in the promoters of their target genes, activating their transcription. From Miklossy et al., 2013.

STAT proteins promote fundamental cellular processes, including cell growth and

differentiation, development, apoptosis, immune responses and inflammation. STAT1 signaling is activated in response to interferon (IFN) stimulation and supports immune function partly by controlling the growth and apoptosis of immune cells. Furthermore, STAT1 deficiency provides malignant cells with a growth advantage and leads to increased tumour formation, thus suggesting that STAT1 has a tumour-suppressive function (Adamkova et al., 2007). STAT2 signaling is important for the induction of antiviral effects, moreover, evidences further suggest that altered STAT2 signaling may partly contribute to carcinogenesis through the up-regulation of interleukin-6 (IL-6) production, which promotes STAT3 activation (Gamero et al., 2010). STAT3 function is essential for early embryonic development, moreover, aberrant STAT3 signaling promotes tumourigenesis and tumour progression partly through deregulating the expression of critical genes that control cell growth and survival, angiogenesis, migration, invasion or metastasis (Yue and Turkson, 2009). STAT4 is a crucial mediator of IL-12 function that regulates the differentiation of TH1 cells and their inflammatory responses. Accordingly, STAT4 signaling is associated with autoimmune diseases (Lovett-Racke et al., 2011). STAT5 has two isoforms, STAT5A and STAT5B, which are encoded by distinct genes and share 96% sequence homology, with notable differences occurring in the transactivation domain. STAT5 signaling is important in mammary gland development and milk production, and in haematopoiesis, moreover, it is widely recognized that STAT5 and STAT3 share similar functions in promoting cancer, including the induction of pro-proliferative and anti-apoptotic genes (Buitenhuis et al., 2003). STAT6 signaling is induced by IL-4 and IL-13 and supports immune function, notably regulating the balance between inflammatory and allergic immune responses. Beyond the immune system, STAT6 signaling promotes luminal mammary epithelium development and is implicated in the pathology of lung and airway disease (Chapoval et al., 2011).

Experiments performed on genetically manipulated mice generally suggested a high degree of specificity for the various Stat proteins in individual signaling pathways. The analysis of mice lacking one or more Stat genes has in fact shown relatively discrete phenotypes, assigning each Stat protein to a relatively specific pathway. However, unlike all other members of the Stat gene family, ablation of Stat3 leads to embryonic lethality (Takeda et al., 1997). This finding, along with evidence of its activation by a wide variety of cytokines, growth factors, and other stimuli, implied that Stat3 might be more generally deployed than its relatives and has led to the suggestion that it might represent a primordial Stat protein (Levi and Lee, 2002).

1.1.1 STAT3 canonical signaling pathway

1.1.1.1 Molecular mechanism of canonical Stat3 pathway activation

STAT3 was discovered more than 20 years ago as a component of the interleukin-6-(IL-6) activated acute phase response factor (APRF) complex, which has a crucial role in stimulating expression of innate immune mediators in the liver. It was first described for its DNA-binding activity, capable of selectively interacting with an enhancer element in the promoter of acute-phase genes, known as the acute-phase response element. Molecular definition of this factor demonstrated that the same protein is activated by the entire family of IL-6–type cytokines, which signal through gp130 and related receptors. Moreover, Stat3 pathway can also be activated by diverse agents such as epidermal growth factor receptors (EGFRs), fibroblast growth factor receptors (FGFRs), insulin-like growth factor receptors (IGFRs), hepatocyte growth factor receptors (HGFRs), platelet-derived growth factor receptors (PDGFRs), and vascular endothelial growth factor receptors (VEGFRs) (Siveen et al., 2014).

Structurally, Stat3 is similar to other Stat proteins, having a conserved amino-terminus involved in tetramerization, a DNA-binding domain with a sequence specificity for a palindromic IFN-γ–activated sequence (GAS), an SH2 domain involved in receptor recruitment and Stat dimerization and a carboxy-terminus transactivation domain. Like other Stat proteins, Stat3 is activated by tyrosine phosphorylation at a single site close to the carboxy-terminus (Y705), as well as by serine phosphorylation at a site within the transactivation domain (S727) (Hirai et al., 2011).

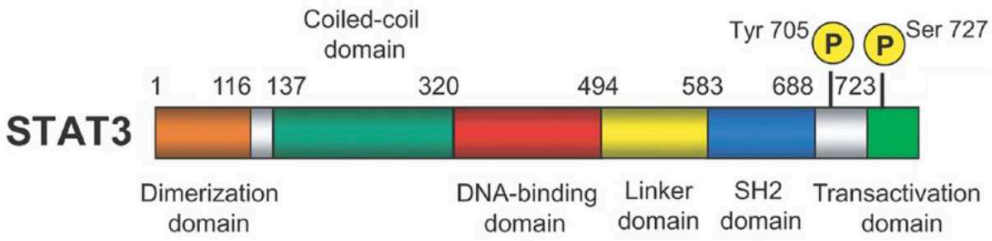

Figure 1.3 Domain structures of mouse Stat3. From Hirai et al., 2011

Given the conservation of the functional domains between STAT3 and the other

members of the STAT family of proteins, the mechanism of pathway activation is consequently similar to the general one previously described.

Specifically for STAT3 protein, the binding of growth factors to the corresponding receptor leads to phosphorylation of receptor tyrosine residues to create docking sites for recruitment of latent cytoplasmic STAT3 through its SH2 domain. The binding of STAT3 at receptor phosphotyrosine sites leads to STAT3 activation through the phosphorylation of tyrosine 705 in the C-terminal domain. Anyway, other classes of non-receptor protein tyrosine kinases have also been reported to stimulate STAT3 activation: specifically, in the case of receptors lacking intrinsic tyrosine-kinase activity, the recruitment of receptor-associated tyrosine kinases, such as JAK and Src, leads to STAT3 phosphorylation via a tyrosine phosphorylation cascade. Interestingly, in transformed cells, STAT3 can also be directly activated by constitutively active non-receptor tyrosine kinases, such as Src (Yu et al., 2007).

STAT3-mediated signaling is typically transmitted through the formation of Tyr705 phosphorylated STAT3 homodimers, whose interaction occurs between the phospho-Tyr705 within one monomer and the SH2 domain within the other. However, in immune cells, the complex cytokine signaling required for generating a robust and specialized immune response is mediated through the use of limited number of STAT molecules, which involves the heterodimerization of STAT proteins. A representative example is found in the IL-6 superfamily of cytokines: in response to IL-6 STAT3 can act both as STAT3:STAT3 homodimers or STAT1:STAT3 heterodimers, which are found especially in late phase of signaling cascade (Guzzo et al., 2010). IL-27, which uses the IL-6 receptor subunit gp130 to induce STAT1 and STAT3 activation cascade, was also shown to induce STAT1:STAT3 heterodimers, although the molecular consequences of that heterodimer are still not clear (Siveen et al., 2014).

These homo-or hetero STAT3 dimers then dissociate from the receptor and translocate to the cell nucleus through the action of Importin α5/NPI-1, that mediates the nuclear transport of the dimers through a STAT3-binding domain in C terminus. In the nucleus, STAT3 dimer binds to DNA, where specifically four loops per monomer contact the sugar-phosphate backbones of both DNA strands. Here they recognize and bind specifically an 8 base pair inverted repeat DNA element with a consensus sequence of 5-TT$(N)_4$AA-3 which is present in the promoter regions of STAT3 responsive target genes (Turkson et al., 1997). This way STAT3 dimers regulate the transcription of genes involved in cell proliferation, differentiation, and apoptosis.

1.1.1.2 Roles of canonical STAT3 pathway

One of the first evidences collected during STAT3 role investigations, was obtained already in 1997 through the generation of a *Stat3* KO mouse. As mentioned before, unlike ablation of other Stat family genes that produces viable mice with relatively limited phenotypes, ablation of Stat3 led to embryonic lethality shortly after blastocyst implantation, between 6.5 and 7.5 days (Takeda et al., 1997). STAT3 appears indeed to be indispensable for embryogenesis in mammals, and even for mouse embryonic stem cells survival (Matsuda et al., 1999). Consistently, *Stat3* mRNA is present in both maternal and extraembryonic tissues during early post-implantation stages of murine development. Furthermore, activated Stat3 protein is present from embryonic days 4.5 to 9.5 in decidual swellings of the visceral endoderm. Because the visceral endoderm plays an important supportive role during early embryogenesis, fostering metabolic exchange between embryo and placenta, it has been hypothesized that Stat3 may be involved in a nutritional process that supports the implanted blastocyst (Levi and Lee, 2002). To date, the process that leads to the activation of STAT3 pathway during early embryogenesis as well as the exact embryonic process mediated by STAT3 remain a mystery.

Interestingly, studies performed in cell culture systems, revealed that STAT3 mediated response to IL-6 cytochines evoke a number of distinct processes in different cells, including induction of an acute-phase response in hepatoma cells, stimulation of proliferation in B lymphocytes, activation of terminal differentiation and growth arrest in monocytes, and maintenance of the pluripotency of embryonic stem cells (Raz et al., 1999).

But how can a single transcription factor be involved in such a broad range of seemingly contradictory cell responses? The answer to this question can be found at least in part in Stat3 capability to induce distinct sets of target genes in different cells. Unfortunately, the early lethal phenotype of the *Stat3* KO model, avoided any further investigation of STAT3 tissue specific roles in adult organs.

The biological effects of STAT3 have thus been evaluated by tissue-specific targeted gene ablation in transgenic mice engineered using a Cre/Lox mediated technology (Akira, 2000).

Stat3 depletion in the epidermis was obtained driving Cre expression under the control of keratin 5 promoter. In these mice the hair cycle and wound healing

processes are severely compromised, displaying sparse hair and spontaneous development of ulcers with age. This results together with others led to conclusion that Stat3 is involved in maintenance of postnatal interactions between epithelia and mesenchymal compartments (Sano et al., 1999). The same transgenic line was also used to evaluate the consequences of Stat3 loss in the thymic epithelium: alterations in this tissue were only detectable in the adult model, which developed severe thymic hypoplasia with loss of thymocytes and normal thymic architecture (Sano et al., 2001). T cells play a central role in cell-mediated immunity. Specific gene ablation using Cre driven by the Lck promoter was used to evaluate STAT3 role in T cells, especially focusing on the consequences on IL-6 pathway, that is known to exert immunomodulatory functions on T cells and stimulation of T cell survival. Consistently, IL-6–mediated T cell survival is impaired in *Stat3*-deficient T cells (Takeda et al., 1999). In macrophages and neutrophils, Stat3 function has been examined by Cre-mediated gene ablation directed by the macrophage lysozyme (Mlys) promoter. Mutant mice display enhanced susceptibility to endotoxic shock and develop chronic enterocolitis with age, which can derive from the inability, in the absence of Stat3, to suppress production of TNF-α once it has been induced during bacterial infection. Furthermore, Mx promoter has been chosen to ablate *Stat3* in the hematopoietic progenitor cells, in order to evaluate its role in granulopoiesis. Surprisingly, all major myeloid cell types develop even in the absence of Stat3, and granulocytes proliferate and differentiate in response to G-CSF (McLemore et al., 2001).

In the mammary gland, STAT3 phosphorylation occurs during the involution process, and in fact, *Stat3* deficient mice show a delay in the onset of involution, suggesting a pro-apoptotic role for Stat3 in this gland. Notably, STAT3 role in the mammary gland appears to be the opposite with respect to its role in the thymus (Chapman et al., 1999). Stat3 is known to be activated in the brain, in particular, it is required for the neurotrophic effects of ciliary neutrophic factor (CNTF) and LIF on developing sensory neurons *in vitro*; this is consistent with the finding that few of these cells survive in mice with selective depletion of Stat3 gene in the brain, resulting in perinatal lethality (Alonzi et al., 2001). A similar survival function has been found for Stat3 in injured motoneurons. Finally, Stat3 deficient neurons are unable to induce Akt phosphorylation in response to CNTF, suggesting a direct link between activation of Stat3 and of Akt.

IL-6 dependent induction of the acute phase response in the liver during inflammation has also been linked to Stat3: in the absence of Stat3, in fact, genes exclusively regulated through Stat-binding sites are silent, while those dependent on other transcription factors, including CAAT/enhancer binding protein (C/EBP) family members and NF-κB, are affected to a lesser extent. These results clearly demonstrate the fundamental importance of Stat3 transcriptional activity to the cytokine-mediated induction of acute-phase response genes. Interestingly, this is the only capacity in which Stat3 function in vivo can be clearly ascribed to its activity as a transcription factor (Alonzi et al., 2001b). Finally, STAT3 activity is absolutely required for small-intestine crypt stem cell survival at both the +4 to +6 label-retaining and crypt base columnar cell locations in mice (Matthews et al., 2011).

To conclude, several evidences showed that Stat3 function as a transcriptional activator can lead to the most different biological readouts such as proliferation, survival, or apoptosis, depending on the target tissue. Moreover, in some cases its activity seems to be independent on its transcriptional activity, identifying it as a transcriptional repressor or as a signaling adaptor.

1.1.1.3 STAT3 role in the intestine

The intestinal epithelium represents the most vigorously renewing adult tissue in mammals, undergoing complete cell renewal every 5 days along the whole life of the mouse (Barker et al., 2008). This process is fuelled by the adult intestinal stem cells, which in the murine model populate the base of the intestinal crypt and divide to generate the crypt progenitors Transient Amplifying cells (TA). TA cells proliferate every 12–16 h, generating some 300 cells per crypt every day that reach the crypt–villus junction, where they rapidly and irreversibly differentiate. Thus, the epithelial sheet is in a continuous upward movement, in which epithelial cells produced in the lower part of the crypt migrate up onto an adjacent villus in a coherent column. According to the more recent model, that is based on the idea of a whole stem cell zone at the base of the intestinal crypt, the “true stem cells” are the so called Crypt Base Columnar (CBC) cells: small undifferentiated cycling cells hidden between the Paneth cells. This model was less popular than the older “+4 position” model, proposed in the late 1950s, which assumes that that the crypt base is exclusively

populated by terminally differentiated Paneth Cells and the stem cells must therefore be located just above the Paneth cells at the +4 position (Barker et al., 2008).

To date, a definitive model assumes the existence of both CBC and +4 cells in the intestine of the mouse model: while CBC are exclusively expressing Lgr5+ and robustly dividing to generated all types of differentiated intestinal epithelial cells, the +4 cells are almost quiescent but may revert back to robustly dividing Lgr5+ stem cells upon crypt damage (Fujii and Sato, 2014).

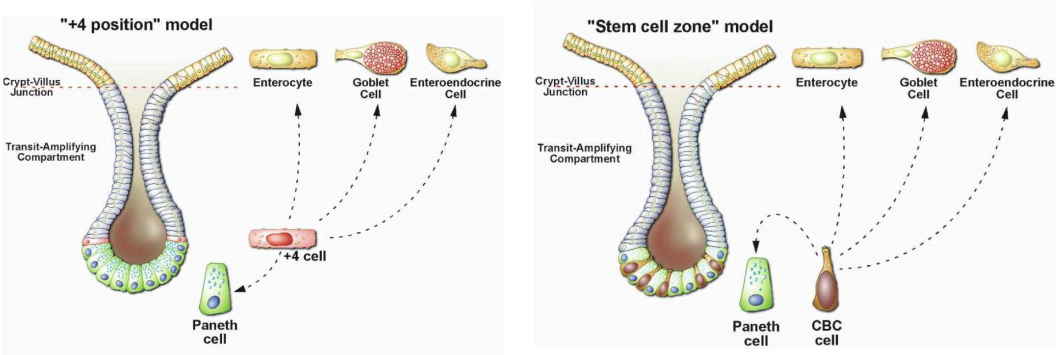

Figure 1.4 The two models for the localization of intestinal crypt stem cells. In the"+4 position"model it is assumed that the crypt base is exclusively populated by Paneth Cells with the stem cells located just above at the +4 position**.** The "stem cell zone" model states that small, undifferentiated, cycling cells, termed crypt base columnar cells, intermingled with the Paneth cells likely are the true intestinal stem cells. From Barker et al., 2008.

Interestingly, although no universal marker for the two intestinal stem cell candidates has to date been identified, evidences obtained on mice demonstrated the absolute requirement of STAT3 for survival of both +4 label-retaining and crypt base columnar cells in the murine small intestine (Matthews et al., 2011). Specifically, they observed that crypt stem cells transiently lacking Stat3 exit the pluripotent/self-renewing state, proliferate, and get removed through apoptosis. More recently Stat3 was found to be indispensable for intestinal *in vitro* organoids formation and to mediate organoids regeneration downstream IL-22 input (Lindemans et al., 2015).

To date, only these few evidences have been collected concerning the role of Stat3 in intestinal homeostasis maintenance, leaving an open question on the direct biological function that Stat3 exerts into ISC.

1.1.2 STAT3 in cancer

Multiple lines of evidence place STAT3 at a central node in the development, progression, and maintenance of many human tumours, validating STAT3 as an anticancer target.

In contrast to the transient nature of STAT3 activation in normal cells, in fact, persistent activation of STAT3 has been reported in a variety of human tumour cell lines and primary human tumours, including leukemias, lymphomas, multiple myeloma, glioma, melanoma, head and neck, breast, ovarian, endometrial, cervical, colon, pancreatic, lung, brain, renal, and prostate cancers (Siveen et al., 2014).

Historically, the potential oncogenic role of STAT3 was established by the expression of constitutively activated STAT3 in various tumour cell lines: cancer cells harbouring aberrant STAT3 activity have elevated levels of anti-apoptotic (Mcl-1 and Bcl-xL) and cell cycle regulating proteins (cyclin D1 and c-Myc). Thus, cancer cells expressing constitutively activated STAT3 are more resistant to apoptosis and chemotherapies aimed at initiating apoptosis. Importantly, this abnormal presence of p-STAT3 is never due to mutations at the *STAT3* gene, but instead, arises from oversupply of growth factors or cytokines within the tumour microenvironment that activate STAT3 in a paracrine manner.

Under normal conditions, STAT3 signaling is tightly regulated by several inhibitory molecules, including the suppressor of cytokine signaling (SOCS), SH2 domain-containing phosphatase-1 (SHP-1) and SHP-2, and protein inhibitor of activated STAT (PIAS) proteins.

However, many documented events were shown to directly trigger STAT3 activation or the release of inflammatory mediators as part of an autocrine pathway; among those are the activation of oncogenes, inactivation of tumour-suppressor genes, chromosomal rearrangement/amplification, deregulation of multiple potential upstream inputs such as elevated EGFR expression levels, EGFR mutations that result in constitutive RTK activation, overexpression of Src, mutations that hyperactivate JAKs and other genetic events in neoplastic cells (Siveen et al., 2014). Moreover, hyper-activation of STAT3 can also result from mutations in any of those negative regulatory proteins, which in normal cells function by limiting the extent of STAT3 activation. One of them is for instance SOCS3, whose epigenetic silencing by hypermethylation in its promoter CpG islands was seen to induce Stat3-mediated cell proliferation and survival (He et al., 2003).

Downstream the constitutive activation of STAT3 have been identified many cellular processes which favour tumour initiation and progression, including proliferation, survival, inflammation, invasion, metastasis and angiogenesis; indeed, tumour with elevated levels

of activated STAT3 have been associated with a poor prognosis (Johnston and Grandis, 2011).

Activated STAT3 promotes proliferation primarily by stimulating transcription of key cancer genes linked with proliferation of tumour cells, such as cyclin D1, cyclin B and cdc2, which are involved in the regulation of cell cycle. In normal tissues, cell proliferation is controlled by irreversible entry into post-mitotic differentiated states. Emergent tumour cells escape this terminal differentiation by over expressing c-Myc, a known Stat3 target, found at 5 folds elevated levels in transformed cells. Nevertheless, several anti-apoptotic proteins, such as survivin and members of the Bcl family (Bcl-xL, Bcl-2 and Mcl-1), which are known to be crucial for tumour cell survival, are direct target genes of STAT3.

However, Stat3 does not contribute to the tumourigenic process only by triggering pro-survival and pro-proliferative signaling, it is in fact the major intrinsic pathway for cancer inflammation, often activated in tumour-associated immune and inflammatory cells as well as in malignant cells. Being capable of inducing a large number of genes that are crucial for inflammation, including IL-6, 10, 11, 17, 23, CXCL12 and COX-2, Stat3 is crucial for the initiation and maintenance of a cancer-promoting inflammatory microenvironment. Within the tumour microenvironment, an inflammatory loop is created by the persistent activation of STAT3 in tumour cells, which leads to the production of cytokines, chemokines and growth factors. Once in the extracellular space, these molecules associate to the receptors of stromal inflammatory cells activating Stat3 pathway and causing the release of more inflammatory mediators to the microenvironment (Yu et al., 2014). In particular, IL-6/STAT3 mediated chronic inflammation exhibits a strong association with many types of tumours, including gastric and colorectal cancer (Ernst et al., 2008).

In conclusion, being ectopically expressed in nearly 70% of solid and haematological malignancies, STAT3 represents a promising therapeutic target for many different tumours.

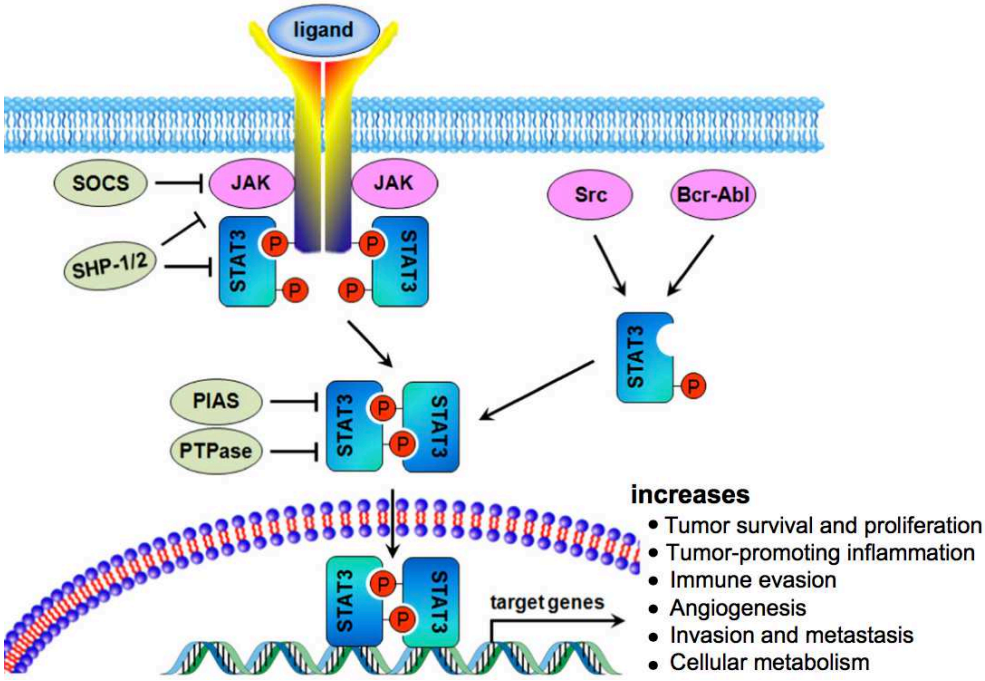

Figure 1.5 Schematic representation of the proteins involved in Stat3 pathway regulation during tumourigenesis. The STAT3 signaling is activated by binding of ligands to their corresponding cell surface receptors that leads to activation of receptor-associated JAK kinases by phosphorylation of tyrosine residue. These in turn phosphorylate STAT3 that can also be directly phosphorylated by non-receptor tyrosine kinases such as Src and Bcr-Abl. Phosphorylated STAT3s homodimerize and translocate to the nucleus where it regulates gene transcription. STAT3 signaling is negatively regulated by SOCS and PIAS family of proteins, as well as by SHP-1/2 and protein tyrosine phosphatase (PTPase). From Kim et al., 2016.

1.1.2.1 STAT3 in Colorectal Cancer

As mentioned before, Stat3 is known to promote colitis-associated colon cancer by inducing and maintaining a cancer-promoting inflammatory microenvironment (Yu et al., 2014). However, many colon cancers arise from somatic mutations in the tumour suppressor gene *APC* (adenomatous polyposis coli), which cause constitutive activation of the Wnt/β-catenin pathway in the intestinal epithelium (Phesse et al., 2014). The Wnt/β-catenin pathway is, similarly to the JAK/STAT3 pathway, involved in intestinal homeostasis maintenance, proliferation of the epithelial stem cell compartment at the base of the intestinal crypt and required for intestinal regeneration. Consistently with that, experiments aimed at investigating the functional interaction between JAK/STAT3 and Wnt/β-catenin signaling pathways in

Apc-induced mouse CRC, revealed that Stat3 is required for intestinal tumourigenesis and tumour growth caused by loss of APC. Interestingly, however, no direct interaction between the two pathways has to date been elucidated, since inhibition of JAK/STAT3 signaling does not affect the accumulation of β-catenin in *Apc*-mutant tumours. STAT3 pathway was seen to be rate-limiting for the tumour growth through control of the Bmi1 gene, which acts as a repressor of cell cycle inhibitors p21 and p16. Stat3 depletion, by abolishing Bmi1 expression, leads to accumulation of cell cycle inhibitors, thus blocking the proliferation of tumour cells. Moreover, C-myc oncogene is also known to be a target of Stat3, thus promoting proliferation under Stat3 inputs.

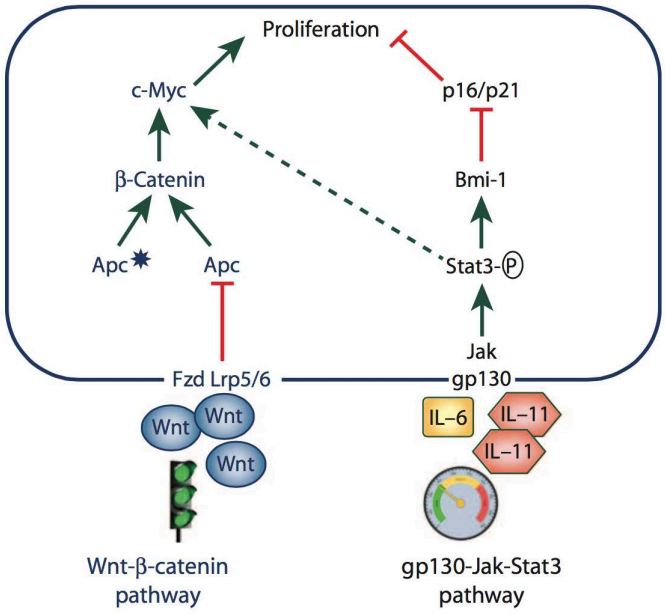

Figure 1.6 Schematic representation showing how the Jak/Stat3/Bmi-1 pathway acts to regulate Wnt/β-catenin–activated cell proliferation (*Apc, mutated *Apc*).** Stat3 pathway acts in a parallel manner to Wnt/β-catenin regulating cell proliferation through activation of pro-proliferating c-Myc and inhibition of cell cycle negative regulators p21 and p16 by Bmi1 expression. From Phesse et al., 2014.

This suggests that the JAK/STAT3 pathway serves to fine-tune the proliferative response of the intestinal epithelium during *Apc*-induced CRC.

Importantly, experimental evidences collected in the same paper by Phesse et al., showed that the partial inhibition of the JAK/STAT3 signaling, which is sufficient for

the inhibition of tumour growth, is not affecting intestinal homeostasis in normal tissue, corroborating the idea of STAT3 as an amenable therapeutic target for CRC.

1.1.3 STAT3 role in mitochondria

During the past 15 years there have been a number of reports suggesting that a variety of nuclear transcription factors (TFs) resides in the mitochondria, including NFκB, p53, AP-1, CREB, MEF2D, and IRF-3. Since its discovery twenty years ago, STAT3 has been studied extensively, and to date its function of transcription factor, responsible for controlling a diverse array of biological functions, has been well-characterized. However, the recent finding of a pool of Stat3 localizing to mitochondria, has opened a new area to discover non-classical functions.

mitoStat3 was first identified by Wegrzin et al. in 2009, as a positive regulator of the ETC. Afterwards, a number of follow up studies have further confirmed the presence of Stat3 in mitochondria in different cells and tissues.

Several studies observed that GRIM-19, a component of Complex I of ETC, binds to Stat3 directly, and in fact, mitoStat3 was found associated with the inner mitochondrial membrane where the Complex I resides. However, as with most of mitochondrial proteins encoded by nuclear genes, the mechanisms by which Stat3 is transported to the mitochondria remain unclear. Although the C-terminus of Stat3 has been shown to be required for mitochondrial transport, no specific mitochondrial targeting signal has been identified (Yang and Rincon, 2016). Most likely, Stat3 association with GRIM-19 leads to mitochondrial targeting through the TOM-20 importer; however, a second import mechanism involving the Hsp22 protein has been suggested.

Several evidences suggest that mitoStat3 capacity to increase the electron transport chain efficiency, thus influencing mitochondrial ATP synthesis, passes through its direct binding to complex I and II. Coherently, Stat3 deficiency impairs complexes function, which can indeed be rescued in *STAT3-/-* cells by a mitochondrial-targeted form of STAT3 (MLS_STAT3) (Wegrzin et al., 2009).

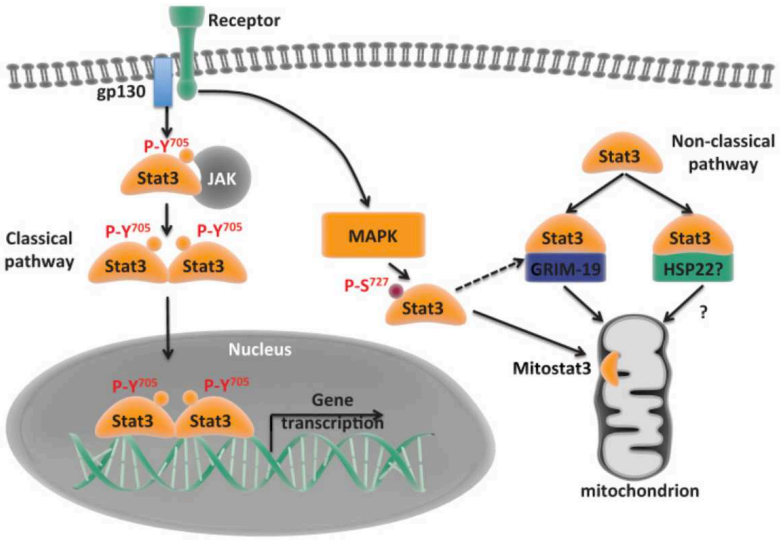

Figure 1.7 Classical and non-classical pathways of Stat3. Classical pathway where Stat3 translocates to the nucleus and mediates gene transcription. Non-classical pathway where Stat3 is recruited to mitochondria and regulates functions alternative to transcription. From Yang and Ricorn, 2016.

Consistently with the knowledge that complex I largely contributes to establish the mitochondrial membrane potential (MMP), which is primarily used for ATP synthesis by Complex V of ETC, lack of mitoStat3 was reported to lower the levels of ATP in both astrocytes and cells undergoing oncogenic transformation (Gough et al., 2009). Interestingly, however, the increase in mitoStat3 does not result in any change of ATP levels in CD4 T cells, suggesting a tissue and cell specific regulation of ATP synthesis by mitoStat3 (Yang et al., 2015). Biochemical studies aimed at unravelling the molecular mechanism behind mitoStat3 regulation of cellular respiration, discovered that although both Tyr705 and Ser727 phosphorylations has been found in mitoStat3, p-Tyr705 does not seem to be required for the optimal activities of ETC in several cell types. In contrast, Ser727 phosphorylation results critical for mitoStat3 to enhance ETC Complex I and Complex II activities. Studies performed with two mutated forms of murine MLS-STAT3 (Y705F, S727A) in STAT3-/- cells clearly showed that complex I activity could only be rescued in the presence of a S727 phosphorylation site, independently on Y705 phosphorylation. Moreover, in the same paper, Wegrzin et al. suggest the that a monomeric mitoStat3 binds Complex I and promote ETC activity, since a constitutively dimeric form of Stat3 could not rescue complex I defects in STAT3-/- cells.

In addition to the regulation of mitochondrial ATP production, very recently, it was demonstrated that mitoStat3 also plays a role in Ca2+ homeostasis (Yang et al., 2015) and in ROS production, even though controversial findings have been published to date about this last mitochondrial function (Yang and Ricorn, 2016).

Although the first evidences collected on mitoStat3 excluded the possibility for it to act as a regulator of mitochondrial genes expression (Wegrzin et al.,2009), more recent evidences showed the direct binding of Stat3 protein on mtDNA in keratinocytes culture (Marcias et al., 2014). Interestingly, the d-loop transcriptional promoter in mitochondrial DNA contains several Stat3 responsive elements, similarly to the promoters of nuclear Stat3 target genes. Finally, in mouse ESC cultures, mitoStat3-dependent transcriptional regulation of mitochondrial genes, was shown to promote proliferation and induce naïve pluripotency (Carbognin et al, 2016).

1.2 Zebrafish as a model organism

Danio rerio, better known as zebrafish, is a tropical vertebrate fish, belonging to the family of Cyprinidae and original of the Ganges region in eastern India. It commonly inhabits streams, canals and other slow-moving or stagnant water bodies but it is also widely sold as an aquarium fish.

Ever since George Streisinger pioneered his research using zebrafish in Eugene, at the University of Oregon in 1972, it has become a great animal model both in basic and biomedical research. No other vertebrate model organism's popularity has grown as quickly as that of the zebrafish: its reputation was firmly established during the nineties of the 20th century when big genetic screening projects in US and Europe led to the discovery of thousands of mutants that gave novel insights into the functions of essential human developmental genes. Being vertebrate, zebrafish share a high degree of sequence and functional homology with mammals, including humans. Due to the conservation of cell biological and developmental processes across all vertebrates, studies in fish can help understanding human disease processes. To this point, it is notable that all proteins that have to date been studied, appear to have similar function in fish and mammals.

However, the genetic similarity to mammalians is not the only relevant reason to choose zebrafish as research model: a leading motive is for sure the optical transparency of zebrafish embryos which makes them ideally suited for microscopic imaging of live processes. Fluorescent markers can be also used to 'light up' different cells and organs in the embryo giving the possibility to follow the impact of a genetic manipulation or pharmacological treatment using non-invasive imaging techniques.

Importantly, many simple and highly efficient genetic manipulation techniques have been developed that allow targeted mutagenesis or generation of transgenic lines; moreover, Zebrafish embryos are able to absorb many chemicals by simple dissolution of them in the embryo medium, thus representing a useful tool for drug screenings.

Due to their small size and the simplicity of their needs, it is easy to keep zebrafish by mimicking their natural conditions, thus minimizing the experimental variations and other sources of animals stress. Finally, zebrafish give a large number of offspring in each spawn (300 embryos per mating) that develop relatively fast, ensuring a big and ready supply of animals for research purposes.

1.2.1 Zebrafish reporter lines: in vivo tools to study gene expression

The knowledge of molecular and biochemical processes regulating cell proliferation, differentiation, survival, and death is the basis for understanding animal development, tissue homeostasis and tumour growth (Moro et al., 2013). These biological functions are known to be regulated by embryonic and postnatal signaling pathways, that are indeed often disrupted in cancer and diseases. Among those are the bone morphogenetic proteins (BMPs), Notch, wingless-related integration site (Wnt), sonic Hedgehog (Shh), fibroblast growth factor (FGF), signal transducer and activator of transcription (STAT), activator protein 1 (AP1) and cAMP-response element-binding protein (CREB) signaling pathways (Perrimon et al., 2012). These pathways rely on signal transduction cascades that culminate in the transcription of arrays of genes driven by specific set of transcription factors. The dissection of the precise logic behind the transcription activation moved by a specific pathway, has led to the identification of cis-elements in the promoters of relevant target genes and the subsequent isolation of the TFs binding to these cis-elements. The generation of synthetic constructs bearing multimerized copies of a specific cis-element bound to a minimal promoter of a reporter gene, led to the development of transgenic animal reporter lines expressing *in vivo* fluorescence reporter proteins under the control of signaling pathway responsive elements. This modern approach has been applied to almost all model organisms, from mice to Drosophila and more recently to zebrafish, representing an innovative and reliable tool to investigate *in vivo* the spatial and temporal activation of a specific pathway. Specifically, the transparency of the zebrafish embryo together with the possibility to follow early development and organogenesis, due to external fertilization, renders this model an ideal tool for analytical screenings and analysis of pathway reporters.

1.2.1.1 Generation of zebrafish transgenic reporter lines

According to the 2013 review by Moro et al., which attempts to give an overview on the transgenesis technique at the base of reporter lines generation in zebrafish, four major critical issues have to be considered: the choice of a suitable transgenesis method; the design and optimization of multimerized signaling responsive elements; the use of a non-tissue specific and strong minimal promoter and the selection of an appropriate reporter protein (e.g. GFP, DsRed, mCherry, Kaede, Caerulean, YFP) (Moro

et al., 2013).

Since the first successful attempt to introduce foreign DNA into the zebrafish germline (Stuart et al. 1988), considerable advances have been made in the field especially after the development of the Tol2 technology by Kawakami et al., in 1998, which to date represents the most widely used transgenesis method for aquatic vertebrates.

This method relies on the microinjection into fertilized eggs of a transposon-donor plasmid, and a synthetic mRNA encoding the Tol2 transposase, to catalyse DNA excision and recombination within the targeted genome (Moro et al., 2013). The donor vector is generated by assembling the required components of the responsive transgene into a final destination construct, most of the times taking advantage of a Gateway-based Tol2 strategy (Kwan et al. 2007). This kit ensures the quick, modular assembly of a [promoter]–[coding sequence]–[polyA] construct into a Tol2 backbone, basing on the att site-specific recombination system from lambda phage (Hartley et al., 2000).

The responsiveness of a transgenic construct to a particular signaling pathway is dependent on the choice of the right regulatory elements to be located upstream the minimal promoter and coding sequences of the reporter protein. Pathway specificity and sequence information of these responsive elements can be easily obtained from literature, or identified by *in silico* analysis, taking advantage of ad hoc bioinformatics platforms. These tools are classically based on the comparison between the promoter regions of co-expressed target genes, and are designed to detect evolutionarily conserved elements shared among different species (Loots 2008). In order to enhance the efficiency of the TFs in binding to the specific transgenic construct, responsive elements are synthetized in tandem repeats; moreover, evidences collected by Moro et al., demonstrated that the number of responsive sequences in the promoter region of the transgenic cassette, affects the levels of reporter protein expression positively.

As mentioned before, of great relevance is also the choice of the right fluorescence protein. Even though it might be believed that stronger is better, when addressing a biological question dealing with rapid tissue or cell dynamics, a destabilized reporter protein instead of a stabilized or enhanced one is generally preferable. On the other hand, in experimental tests involving analysis of cell signaling dynamics or cell lineage tracking over a long time range, the use of long half-life fluorescent proteins is suggested (Moro et al., 2013).

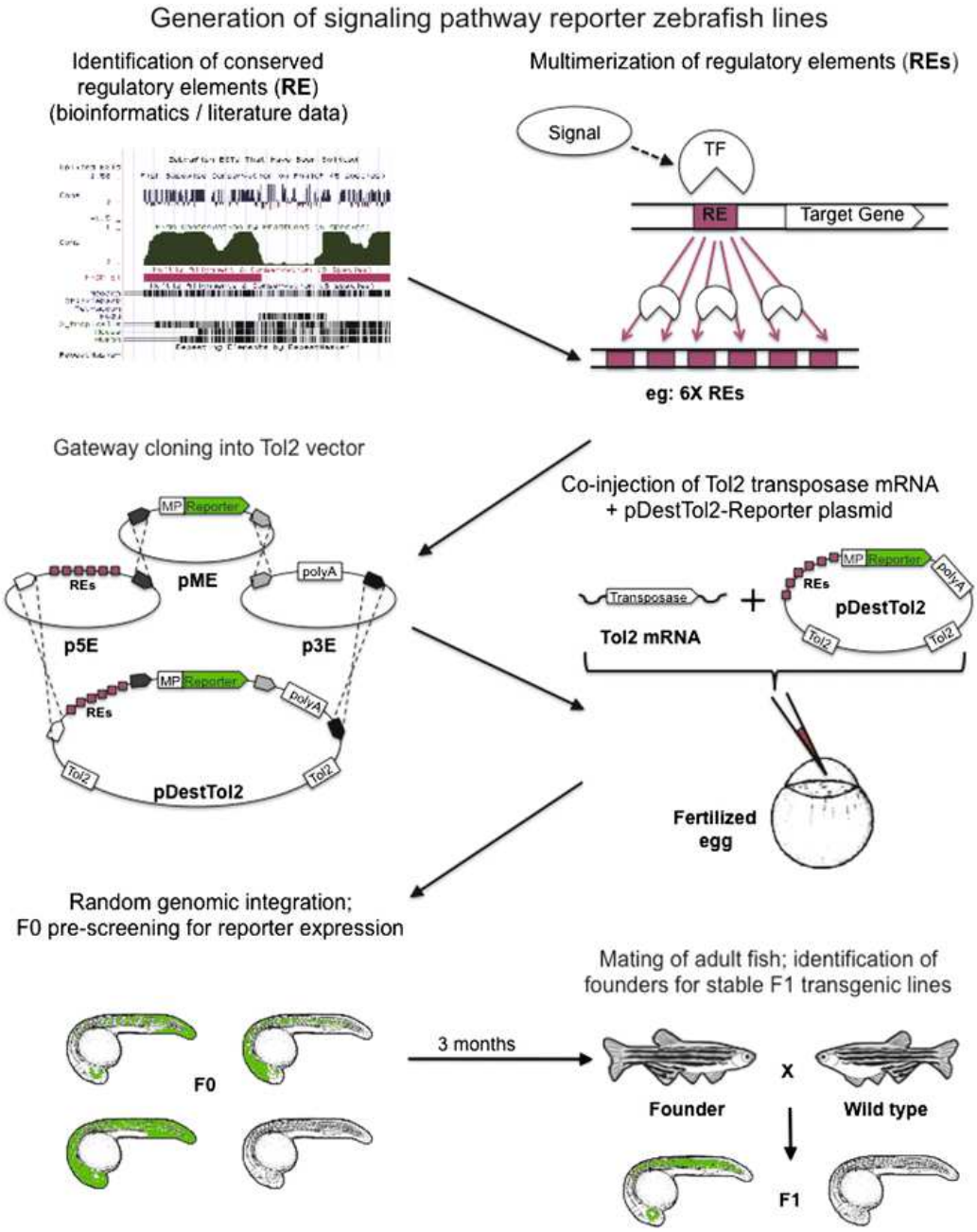

Figure 1.8 Generation of a zebrafish signaling pathway reporter line by the Tol2 strategy. See text for details (From Moro et al., 2013).

Finally, after generation of the desired transgenic reporter construct, microinjection must be performed into 1-2 cell stage embryos, together with transposase mRNA.

F0 injected fish have then to be grown to adulthood and after outcrossing them with WT partners, a screening for fluorescent progeny can be performed in the F1, in order to identify and isolate reporter founder fish.

1.2.1.2 Validation of a signaling pathway reporter line

Before using a zebrafish reporter line for any biomedical application, it is of great relevance to carry out rigorous analysis and validation protocols to establish that the transgenic line is effectively reporting the activity of the desired pathway.

The validation of a reporter line is based on experimental observations and tests aimed at showing that the signal detected is a reliable readout of the signaling pathway activity. There are four major approaches to validate a novel zebrafish reporter line: the comparison of multiple reporter lines, the literature review, the genetic analysis, and the pharmacological screening (Moro et al., 2013).

A first indication of the consistency of the reporter is easily obtained by the comparison of the fluorescence pattern between different founder fish: positional effects and the presence of multiple transgenic alleles in the same carrier can in fact cause possible discrepancies in the reporter protein expression pattern and intensity.

It is important thus to identify founders with single transgenic insertions or to dilute the copies of the transgenic cassette through repeated outcrossings with WT fish.

In the fortunate case of the existence of another reporter line for the same pathway, designed on different responsive element, the comparison between the spatio-temporal activation of the two signals might be useful to assess reliability.

The third approach during the validation process is based on literature research: observing that the reporter signal is present in tissues where the pathway is known to be active, is generally a good indication of the consistency between the signal and the endogenous activation of the pathway.

Finally, probably the most consistent indication of the responsiveness of a zebrafish transgenic reporter line, relies on pharmacological and genetic perturbations of the endogenous signaling pathway, which are able to show whether or not the reporter signal is able to describe variations in the activity of the pathway under investigation.

Genetic methods to up- and downregulate the pathway activity include overexpression of mRNAs encoding for WT or dominant-active/negative versions of pathway

components, or even knockdown by antisense morpholino oligos injection. Similarly, signaling reporter lines can be put in mutant background, or crossed with transgenic lines that overexpress a specific component of the pathway under investigation.

On the other hand, pharmacological methods entail the use of drugs represented by known chemical agonist or antagonist, possibly acting at different levels of the signaling cascade (Moro et al., 2013).

To date, the combined use of all the described approaches remains the best way to investigate the reliability of a novel signaling pathway reporter line.

1.2.1.3 Applications and limits of signaling pathway reporter lines

The most intuitive application of novel zebrafish reporter lines, concerns the visualization and the analysis of the physiological activation of the pathway throughout development and adult life in specific tissues and organs (Moro et al., 2013).

The semitransparency of the fish during early development, allows to follow the reporter signal *in vivo* with single-cell resolution during gastrulation, segmentation and organogenesis. Moreover, the availability of unpigmented fish lines prolongs this possibility to juvenile stages and even adulthood, when observations are limited to superficial organs. Nevertheless, reporter expression in adult internal tissues and organs can be visualized by *post-mortem* dissection through direct fluorescence microscopy and/or immunostaining (Moro et al., 2013). Obviously, the simple observation of the reporter fluorescent signal, can be also used under genetic backgrounds that model specific human diseases, to explore the role of signaling pathways in the initiation ad progression of human pathologies.

Notably, the mating of double or multiple transgenic lines can be used for co-localization analysis to investigate crosstalks or epistatic interactions in both physiological and pathological contexts (Moro et al., 2013).

Another obvious and widely diffused employment of zebrafish pathway reporter lines resides in their previously described capacity to report variations in the pathway activity under external perturbations in vivo. Two examples of this are reporters application into regenerative medicine research, or in the even more diffused screenings and validation studies for novel, therapeutically relevant drugs acting as agonists or antagonists of the considered signaling pathway.

To conclude, zebrafish reporters are commonly considered a resource for answering many unsolved biological questions regarding tissue communications and cell dynamics occurring in living vertebrates; however, some limitations have also to be considered. Previously collected evidences in fact demonstrated that several factors, such as technical limitations in detecting reporter expression (which might be under the limit of detection) or persistent expression of stable reporter proteins (due to positional effects or to tissues with extremely low turnover rate), might lead to misleading biological interpretations.

1.2.2 Zebrafish as a model for human diseases

The zebrafish system was originally envisioned to provide important clues to normal embryogenesis and organ development, however, in the last decade, it became an attractive animal system for modelling human diseases. The true usefulness of the model was established as a result of several large forward genetic screens, which identified mutants in almost every organ or cell type. Remarkably, most of the times, these mutant fish share their phenotype with mammals (White et al., 2013). Moreover, fish genome shares a high degree of sequence similarity to that of humans, and approximately 70% of genes associated with human diseases have functional homologues in the fish. Strikingly, the development and function of zebrafish organs are remarkably similar to those of humans (Santoriello and Zon, 2012).

As a non mammalian species, however, zebrafish have certain disadvantages for modelling human disease that include the lack of some mammalian organs (lung and mammary gland), phenotypic discrepancies with respect to humans, caused by orthologous genes, and most importantly, the presence of gene duplications in the zebrafish genome, that results in genes subfunctionalization and neofunctionalization (Santoriello and Zon, 2012).

Besides those issues, the many benefits of zebrafish biology made it an attractive model for researchers, that in the last decade took advantage of the zebrafish technology, to create models, explore disease biology, and to find new therapies.

1.2.2.1 Zebrafish intestine as a model for human small intestine

As mentioned before, the external development and optical transparency of the embryos, made zebrafish a well-established model for the genetic analysis of vertebrate organogenesis; in particular, studies conducted on several different zebrafish intestinal transgenic lines (gut:GFP (Field et al., 2003), nkx2.2a:mEGFP (Ng et al., 2005) have led to achievement of almost complete knowledge on the development and adult homeostasis of zebrafish intestine.

In its mature form, the intestinal epithelium is a dynamic and self-renewing tissue system that encompasses most aspects of cell behaviour. It comprises both rapidly proliferating, undifferentiated and differentiated cells of multiple lineages specialized to perform digestive, absorptive, protective and endocrine functions.

Notably, histological sections revealed that the anatomy and architecture of adult zebrafish intestinal tract closely resemble that of the mammalian small intestine, where the folds are comprised of a single layer of epithelium that rests upon a connective tissue core. In the mammals small intestine, epithelial homeostasis is maintained by balancing the self-renewal of stem cells, progenitor cell proliferation, cell fate-specific differentiation, cell migration and cell death in spatially distinct compartments that are aligned along a directional axis that originates at the base of pocket-like invaginations, called the crypts of Lieberkühn, and terminates at the tips of finger-like projections known as villi (Ng et al., 2005). Differently, in zebrafish, the epithelium at the base of the folds varies in width and lacks crypts of Lieberkuhn; nevertheless, this configuration, in which the proliferative compartment is restricted to the basal portion of the intervillus pocket, resembles the organization of proliferative cells within the mammalian embryonic intestine.

Three of the four principal cell types within the mammalian small intestinal epithelium are present within the zebrafish intestine: columnar-shaped absorptive enterocytes, mucin containing Goblet cells and enteroendocrine cells. As reported for other cyprinid fish, Paneth cells were not identified histologically in any zebrafish intestinal segment (Brugman et al.,2016). Interestingly, no classical Peyer's patches or microfold (M) cells are found, however, within the the middle portion of zerafish villi, cells containing large vacuoles in which luminal contents can be stored have been identified; these might possibly act as M-like vacuolated cells, delivering luminal

contents to scattered immune cells present underneath the epithelial layer (Brugman et al.,2016).

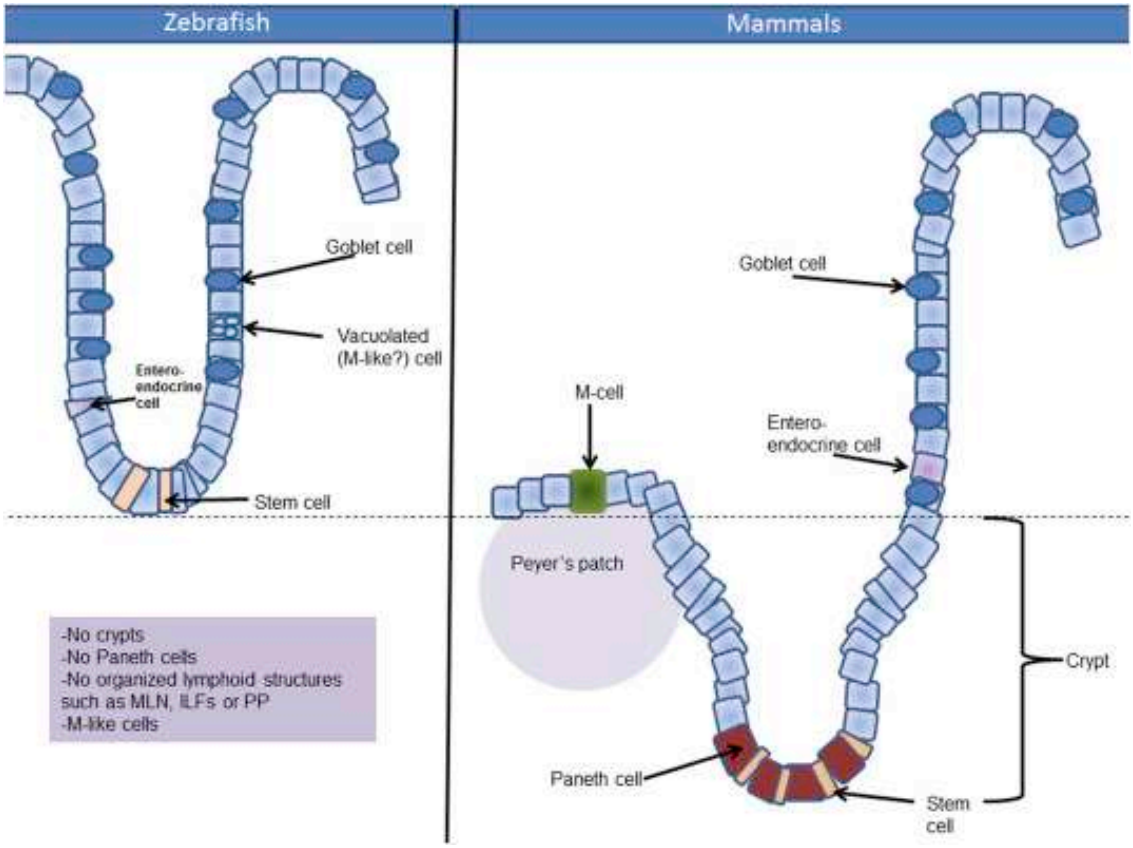

Figure 1.9 Difference in cell types and structures between the zebrafish and mammalian small intestines. Zebrafish do not have Paneth cells, crypts, and organized lymphoid structures such as Peyer's patches (PP). From Burgman et al., 2016.

Notably, expression studies performed on zebrafish intestine revealed that fish intestine is morphologically similar to human intestine and also expresses similar genes. A microarray based study performed by Zhiyuan Gong and colleagues at the National University of Singapore revealed that different segments of zebrafish intestine are transcriptionally similar to different human intestinal portions. Given that zebrafish intestine is divided into three segments (the intestinal bulb, the mid intestine and the posterior intestine), they found that the intestinal bulb and the mid-intestine express genes closely parallel to those of human small intestine, while the posterior intestine expresses genes similar to the caecum and the rectum in the more proximal

portion, while expression is exclusively rectum-like in the more distal portion. Interestingly, no region of zebrafish intestine is exclusively colon-like, while some specific colon genes are found expressed in the intestinal bulb and in the mid-intestine, suggesting that at least some functions of the mammal colon may happen all along their length.

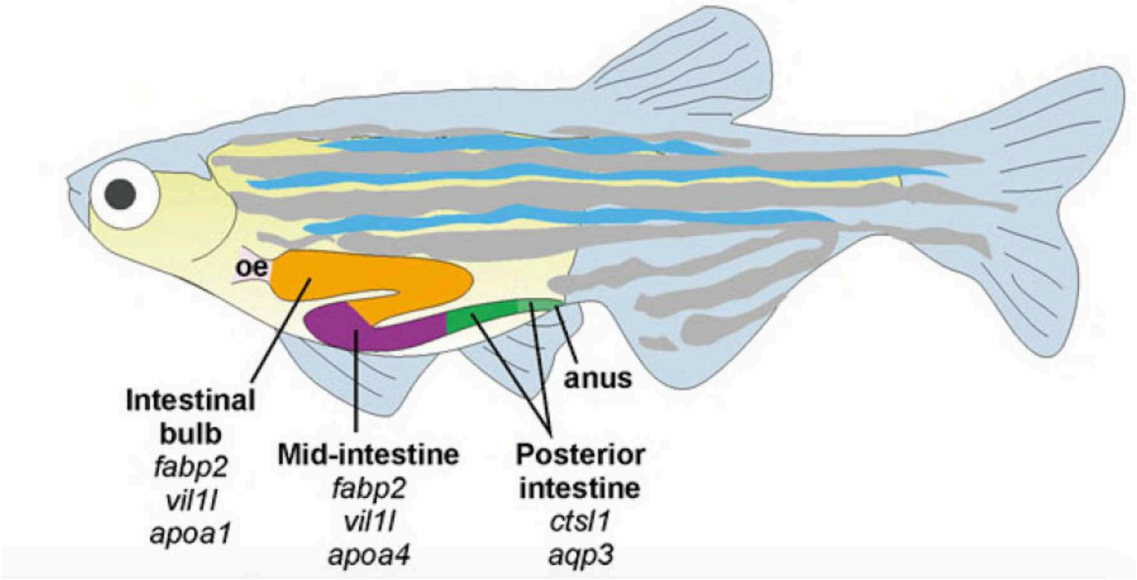

Figure 1.10 Schematic representation of adult zebrafish intestine. Zebrafish intestine is a simple tapered tube that is folded into three sections: the intestinal bulb (orange), the mid-intestine (violet), and the posterior intestine (green). Gene expression in the intestinal bulb and mid-intestine closely resembles that of the mammals small intestine including expression of villin, fabp2, apoa4 and apoa1, indicating roles in lipid and proteins absorption. The posterior intestine expresses genes like acquaporin 3, thus indicating function in water absorption. oe= oesophagus. From 'Cancer and Zebrafish: Mechanisms, Techniques, and Models' by David M. Langenau (2016).

Understandably, morphological and genetic similarities between mammalian and zebrafish intestine led to development of several zebrafish mutants modelling human intestinal diseases including the *apc*- genetic model for colorectal cancer.

1.2.2.2 apc- zebrafish model for Colorectal Cancer

APC (adenomatous polyposis coli) was originally identified as the gene mutated in the familial adenomatous polyposis (FAP) syndrome in humans (Groden et al., 1991).

Humans and mice carrying a truncated form of the APC protein, develop multiple colorectal adenomas following somatic inactivation of the remaining allele.

The Apc protein is a key member of the cytoplasmic β-catenin destruction complex, which operates to provide ubiquitination and degradation of the Wnt pathway TF by the proteasome. In sporadic cases of colorectal cancer, APC mutations cluster in a region proximal to the central Axin-binding motifs, termed the mutation cluster region (MCR). Truncations to the MCR cause the loss of APC function, leading to accumulation of β-catenin and constitutive activity of the Wnt pathway. Similarly to humans and murine model, *apc-/+* fish develop spontaneous tumours starting from 12 months of age, primarily in the liver and subsequently in the intestine of 12% *apc-/+* fish (Haramis et al., 2006).

Importantly, heterozygous loss of APC in the zebrafish appears to phenocopy cancer phenotype of human FAP patients both morphologically and histopathologically, thus validating *apc-/+* fish as a *bona fide* model for human digestive tract neoplasia.

1.3 Aim of the project

Stat3 is one of the most pleiotropic signaling pathways known to date, whose activation is an important event for the cytokine and growth factor-induced cellular and biological processes, including proliferation, differentiation, survival, development and inflammation. Besides the plethora of physiological functions that are known to depend on Stat3, the pathway is also found constitutively active in almost 70% of human solid and haematological tumours, where it acts by increasing tumour cells resistance to chemotherapies. Because of that, Stat3 pathway is of great interest in cancer research, where it represents a promising target for novel therapies.

The aim of this project is the generation, the characterization and the validation of a Stat3 signaling pathway reporter line in zebrafish, with the purpose to plumb Stat3 pathway involvement in zebrafish development, adult organs maintenance and cancer.

Specifically, our scientific interest focuses on the role of Sta3 pathway in the regulation of zebrafish intestinal proliferation in both physiological and pathological conditions, thus modelling human intestine. Moreover, we also approach the novel attractive research field that deals with Stat3 role in mitochondria, thus evaluating the possible involvements of this restricted pool of Stat3 protein in the regulation of proliferation in different tissues.

2 MATERIALS AND METHODS

2.1 Animals husbandry

The fish were staged and fed according to the methods described by Kimmel et al., 1995, and maintained in large scale acquaria systems (Aquarienbau Schwarz, Göttingen-Germany; ZebTech, TECNIPLAST; Müller-Pfleger). A constant high-quality water exchange is guaranteed by recirculation systems with biological filters and a reverse osmosis supply. Water temperature (28,5 °C), pH (neutral) and saline composition (conductivity between 200 and 400 µS) are maintained constant. Before distribution to the tanks, water is sterilized by UV radiation to reduce the infectious agents spreading.

Depending on the number of individuals, the fish are kept in tanks with different volumes, ranging from 1 liter for single animals, to 5-10 liters for up to 30 fish. The dark-light cycle is based on a photoperiod of 12 hours of light and 12 hours of dark. The fish are fed once a day with dry food (TetraMin) and twice with living Artemia salina nauplia. Artemia salina is a primitive aquatic arthropod, bought as cysts and raised to nauplia stage into inverted cone hatcheries, in appropriate salinity, oxygen and light conditions.

The fish are mated in the late afternoon into 1 liter thanks holding an internal removable container with a grid on the bottom. This avoids fish to eat their own eggs and permits to collect the eggs from the bottom of the external tank. Male and female are kept apart by a plastic transparent separator, which is removed in the morning after the light turns on. The eggs are collected with a sieve and transferred in Petri dishes containing embryo medium (fish water), where they are incubated at 28,5 °C (Westerfield, 2000). At 5-8 hpf unfertilized eggs and dead embryos are selected under the dissecting microscope and removed. If they are not fixed or used in some experiments, at 6 days post fertilization (dpf) the larvae are transferred into 1 liter thanks, with the same medium and temperature, and maintained until 2-3 weeks of age, when they are moved to the ZebTech, TECNIPLAST acquaria. From 6 dpf to 2-3 weeks zebrafish larvae are fed once a day with dry Artemia powder (Novotom, JBL).

2.2 Generation of *Tg(7xStat3-Hsv.Ul23:EGFP)* reporter line

The Stat3 responsive promoter sequence, containing the seven tandem repeats from the promoter of the human C-reactive protein (CRP) (nucleotides -123 to -85), was obtained in the form of the pLucTKS3 plasmid, from the Jove Lab. (Department of Pulmonary Diseases, University Hospital Utrecht, Heidelberglaan, 3584 CX Utrecht, The Netherlands). The promoter fragment, containing the Stat3 responsive elements (SRE) and the TK minimal promoter, was isolated from the pLucTKS3 plasmid using HindIII and SacII restriction enzymes. The same restriction sites were then used to finally subclone the fragment into the Gateway 5' entry vector pME-MCS (Invitrogen). The resulting p5E-TKS3 entry vector was recombined with the EGFP-carrying middle entry vector and the p3E-polyA entry clone containing the SV40 late polyA signal (Invitrogen) into the Tol2 destination vector pDestTol2pA2 (Invitrogen) through a MultiSite Gateway LR recombination reaction as previously described (Kwan et al., 2007). 30 pg of the recombined Tol2 destination vectors with 25 pg of in vitro synthesized Tol2 transposase mRNA (Kawakami et al., 2004) were co-injected into one cell-stage wild-type zebrafish embryos. Microinjected embryos were selected for mosaic transgenic expression at 24 hpf using an epifluorescent microscope, raised to adulthood and outcrossed to wild-type fish. The F0 founders were finally selected for fluorescence level and the number of transgenic insertions (i.e. for the proportion of the progeny positive for the transgene) in their genome or in that of the fish of the following generations, in order to establish stable single allele transgenic lines.

2.3 Zebrafish transgenic and mutant lines

The following fish lines were used and outcrossed to the *Tg(7xStat3-Hsv.Ul23:EGFP)* Stat3 reporter line: *apc* ^{hu745} mutant line (Hurlstone et al., 2003), *Tg(HSP70:H2B:mRFP)* transgenic line (not published, Meyer Lab. University of Innsbruck).

2.4 Genotyping of *apc* mutants

apc^{hu745} mutant carriers were genotyped by PCR amplification followed by sequencing for the identification of the point mutation (cytosine to timine). Genomic DNA was extracted from the tail of adult fish and PCR amplified with the following primers: apc-for (5'-CTACCCAACTTTACCTATATCAG-3') and 39 apc-rev (5'-GACTCTCAAAACTGTCAAGGG-3'). The PCR product was sequenced using apc-for primer and the sequence obtained was analysed at position 33 downstream of the sequencing primer.

apc^{hu745} mutant carriers were outcrossed to the *Tg(7xStat3-Hsv.Ul23:EGFP)* Stat3 reporter line, the progeny was screened for the reporter transgene expression at 24 hpf and raised to adulthood. The adult reporter fish were finally screened for the *apc* mutation as described.

2.5 Chemical treatment of zebrafish embryos

The following chemical compounds were used:

- AG490 (T3434 , Sigma);
- LLL12 (573131, Calbiochem);
- LY-364947 (L6293, Sigma);
- Balapiravir (HY-10443, DBA).

All drugs were dissolved in DMSO solvent and stored in small aliquots at -20°C. *Tg(7xStat3-Hsv.Ul23:EGFP)* embryos were decorionated and exposed to the drugs dissolved in fish water with 2 mM 1-phenyl-2-thiourea (PTU) to inhibit pigmentation. 100uM AG490 treatment was performed from 24-48 hpf or from 24-72 hpf: 60uM AG490 was administrated in 3-6dpf treatments. 0,075 uM LLL12 solution was administrated from 24-48 hpf. 60uM LY-364947 solution was administrated from 24 to 48 hpf. A 50uM Balapiravir solution was administrated from 8 hpf to 48 hpf; embryos were decorionated at 24 hpf inside the drug solution. After the treatments, the embryos were either analysed under the epifluorescent microscope or fixed in 4% paraformaldehyde (PFA) in

PBS overnight at 4 °C and then stored in pure methanol at -20 °C for in situ hybridization or P-H3 proliferation assay.

2.6 *In situ* hybridization and quantification

Whole mount RNA in situ hybridization was performed on embryos pre-fixed in 4% PFA in PBS overnight at 4 °C and stored in pure methanol at -20 °C as previously described (Thisse et al., 1993). EGFP probe was synthetized from the pME-EGFP supplied by the Tol2 kit (Invitrogen), which was linearized and transcribed with the T7 RNA polymerase using DIG-labeled ribonucleotides. mt_nd2 probe was obtained by PCR amplification from embryos cDNA using mt_nd2-fw (GCAGTAGAAGCCACCACAAA) and mt_nd2-rv (GGAATGCCGCGGATGTTATA) primers. PCR fragment was then cloned into a pCR2.1®-TOPO® TA vector (Thermo Scientific) according to the manufacturer's instructions, and the riboprobe was transcribed with SP6 RNA polymerase using DIG-labeled ribonucleotides. Riboprobe detection was achieved through NBT/BCIP solution that represents a substrate for the anti-DIG-AP alkaline phosphatase activity producing a visible violet precipitate.

Variations in the intensity of in situ hybridization signal were quantified by subdividing the embryos of the control and the experimental group on the basis of the signal intensity observed under the dissecting microscope. Three subgroups were made (high, medium and low), so that the embryo of the control pool displaying a "high" signal intensity were comparable to the "high" intensity embryos of the experimental pool. The embryos in each subgroup were scored and the data were plotted in a histogram.

Fluorescence in situ hybridization was performed after EdU proliferation assay as described by Thisse et al. publication and staining with FastRed fluorescent dye.

2.7 mRNAs synthesis and injection

pCEP4-Stat3-WT, pCEP4-Stat3-Y705F, pCEP4-Stat3-S727A were a kind gift of Prof. Valeria Poli. 70_pPB-CAG+MLS+ Stat3+NES-pA-pgk-hph-2-2 plasmid containing a murine *Stat3* cDNA flanked by a Mitochondrial Localization Sequence (MLS) and a Nuclear Export Sequence (NES) was a kind gift by Prof. Graziano Martello. The 4 cDNAs were subcloned

into a pCS2+ vector using the In-Fusion® HD Cloning Kit (Clonetech) and the Clonetech online tool for primers design.

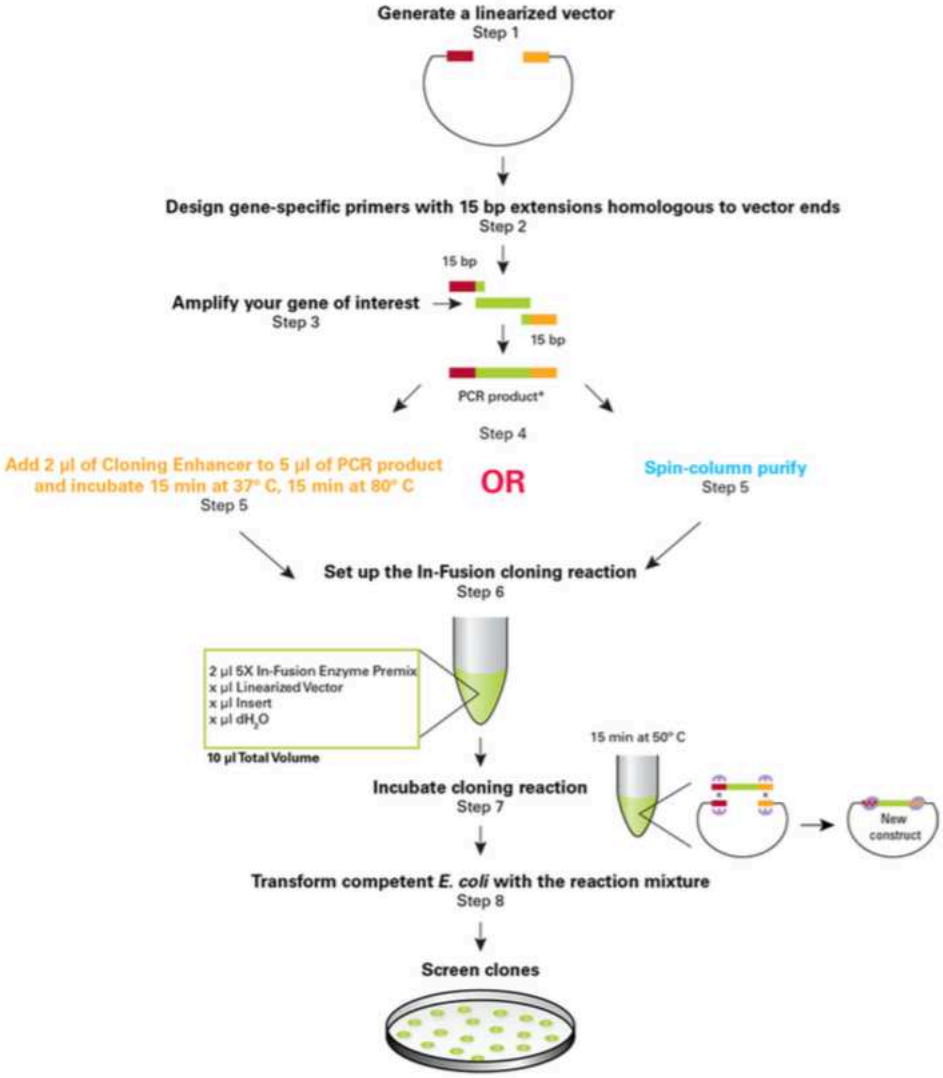

Figura 2.1 Schematic representation of the In-Fusion cloning protocol.

pCS2+-Stat3-WT, pCS2+-Stat3-Y705F, pCS2+-Stat3-S727A plasmids were linearized using NotI restriction enzyme, and mRNA was transcribed using the mMESSAGE mMACHINE® SP6 Transcription Kit (Thermo Fisher Scientific). A mix containing mRNA (30 ng/uL for Stat3-WT, Stat3-Y705F, Stat3-S727A; 50ng/uL for MLS_State_NES) together with the Danieu injection Buffer and Phenol Red injection dye, was injected into 1-2 cell stage embryos. Afterwards the samples were either analysed for fluorescence intensity at

epifluorescent microscope or fixed in 4% paraformaldehyde (PFA) in PBS overnight at 4 °C and then stored in pure methanol at -20 °C for in situ hybridization or P-H3 proliferation assay.

2.8 IL-6 injection

A 50 ng/uL solution of IL-6 recombinant human protein in water (11340064, Immunotools) was injected into the heart of 48 hpf *Tg(7xStat3-Hsv.Ul23:EGFP)* embryos after decorionating and placing them onto a petri dish filled with a thick layer of methylcellulose, to avoid embryos movements. After injections reporter larvae were monitored through epifluorescence microscopy and 3 days post injection the fluorescence variation was reporter using Nikon C2 confocal microscopy. Embryos that displayed reporter signal activation were scored and contingency analysis were performed using Excel software.

2.9 Organs dissection

To analyse the reporter signal in the adult fish, *Tg(7xStat3-Hsv.Ul23:EGFP)* reporters were sacrificed and dissected under the epifluorescence microscope. The intestine and other organs belonging to the gastro urinary tract were imaged immediately after, either *in situ* or isolated, with conventional fluorescence or confocal microscopy. Isolated intestine from *Tg(7xStat3-Hsv.Ul23:EGFP)*/apc+/- adults were dissected in PBS solution and then fixed O/N in 4% PFA in PBS for paraffin embedding and further immunolabeling.
Paraffin embedding and sectioning was kindly performed by Marco Pizzi (Department of Anatomical Pathology, Faculty of Medicine, University of Padua).

2.10 H/E and Alcian Blues staining

Hematoxynin and Eosin (HE) staining on Paraffin embedded intestinal sections was kindly performed by Marco Pizzi (Department of Anatomical Pathology, Faculty of Medicine, University of Padua).

Alcian Blue staining was performed as previously described on the indicated link by Lyons Lab of UCLA (https://www.mcdb.ucla.edu/Research/Lyons/Protocols.html).

Stained sections were observed with Leica DMR microscope and images were acquired using a Leica DFC7000T camera and LAS V4.8 acquisition program.

2.11 Immunofluorescence staining

Immunofluorescence staining on paraffin intestinal sections of adult *Tg(7xStat3-Hsv.Ul23:EGFP)*/apc+/- zebrafish was performed as previously described by Schiavone et al. (2014) using chicken anti-EGFP (A10262, Life Technologies)

(1:250); mouse anti-PCNA (M0879,Dako) (1:100); anti-chicken-Alexa488 (A-11039 ,Life technologies)(1:500); anti-mouse-TRITC (1:500) (R 0270, Dako).

For Immunofluorescence staining 5 dpf larvae were fixed in 4% PFA in PBS O/N and after few preliminary washes in Triton 0,3% in PBS (PBSTr 0,3%), permeabilization was performed Triton 1% in PBS at RT for 30'. Samples were then blocked in 4% BSA in PBSTr 0,3% for 2 hours at RT and then incubated O/N at 4°C with 1° antibody in blocking buffer. The second day samples were washed several times in PBSTr 0,3% and fixed for 2 hours at RT in 4% BSA in PBSTr 0,3%; finally samples were incubated with the secondary antibody in blocking buffer O/N AT 4°C and fixed in 4% PFA in PBS O/N at °C.

2.12 Immunogold labelling and TEM analysis

Immunogold labelling was performed on adult *Tg(7xStat3-Hsv.Ul23:EGFP)*/apc+/- zebrafish intestine using the chicken anti-EGFP (1:25)(A10262, Life Technologies) 1°Ab and a anti-chicken-(10nm)GOLD 2°Ab (25429, EMS). The Electron microscopy service of the Department of Biology (Unipd) performed the immunolabelling and the Transition Electon Microscopy (TEM) acquisitions using a FEI Tecnai G2 microscopy and a TVIPS F114 bottom-mounted camera.

2.13 Heat shock on *Tg(HSP70:H2B:mRFP)*

Adult Tg(HSP70:H2B:mRFP) fish were outcrossed with WTs and the 4 dpf offspring was heat-shocked by replacing the fish water with water preheated to 40 °C and afterwards incubating the larvae in an air incubator at 37 °C for 1 hour. The larvae were then analysed under the epifluorescent microscope for RFP reporter fluorescence, and monitored every 24 hours for the Label Retention Assay with the confocal microscope Nikon C2 H600L.

2.14 stat3Δ8pb mutant generation using Crispr Cas9 mutagenesis

stat3Δ8pb mutant was generated following the optimized protocol for Crispr/Cas9-mediated efficient mutagenesis in zebrafish (Gagnon et al., 2014).

2.14.1 stat3_ex14 sgRNA design

Single guide RNA for Crispr-Cas9 mediated mutagenesis was designed to specifically target exon 14, which is encoding for the central portion of the Stat3 DNA binding domain. This way, Stat3 DNA Binding activity is depleted together with the downstream encoded Transactivation domain, which contains the phosphorylation sites that are necessary for Stat3 functional activation (Fig.2.2).

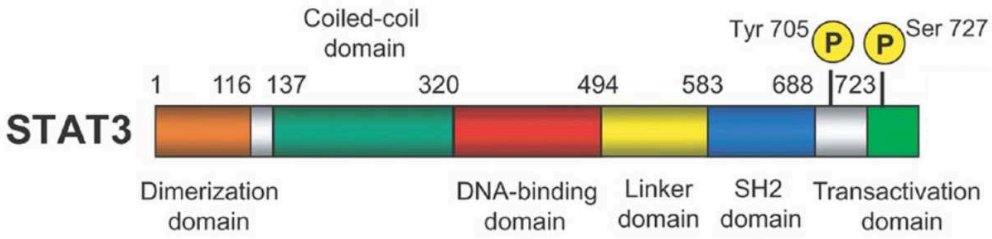

Figure 2.2 Mouse Stat3 functional domains. Representation of the murine Stat3 protein functional domains that are known to be conserved between vertebrate. From Hirai et al., 2011.

stat3_ex14 sgRNA was designed using the free online tool Chop Chop, developed by Harvard University (https://chopchop.rc.fas.harvard.edu/), which allows easy visualization of candidate Cas9 target sites within the gene, and predicts specificity using an algorithm that searches the genome for off-target sites.

Chop Chop output for zebrafish stat3 exon 14, reported in Fig. 2.3, indicated as best target region the GGTCGATCTTAAGTCCTTGGTGG sequence.

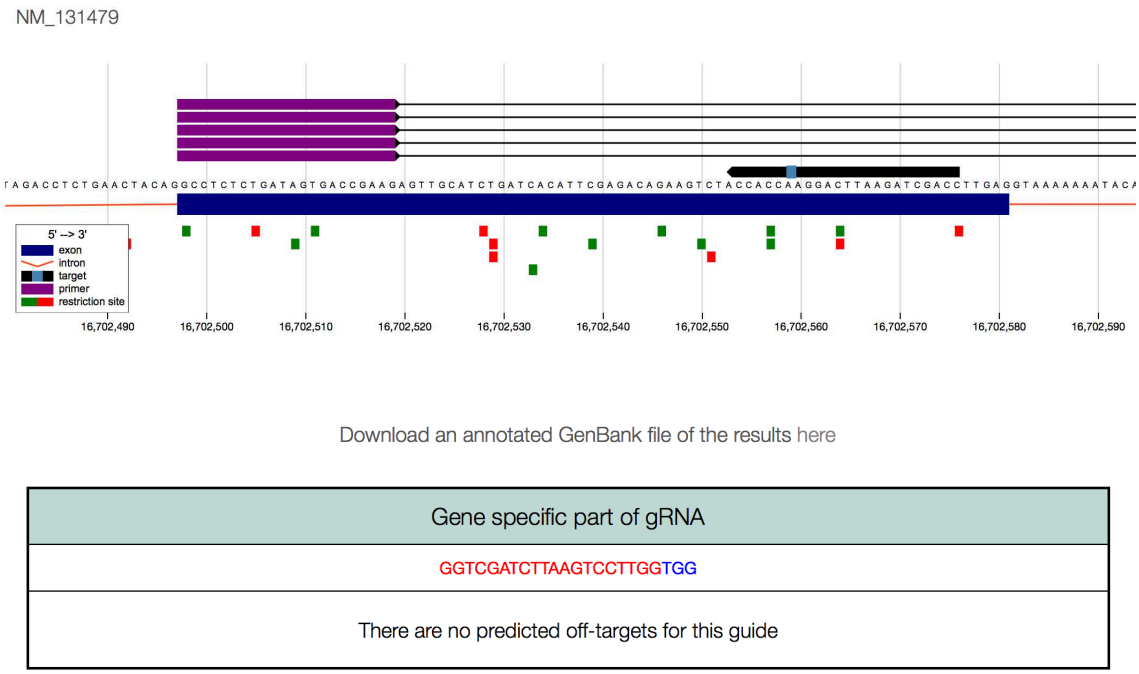

Figure 2.3 Chop Chop output for stat3 exon 14 query target. Chop Chop online tool output indicates the best target region for the sgRNA design and gives a readout of the putative off-targets for it. It also indicates the best primers couples for genotyping with HMA (violet bands) and the restriction sites that might be useful for a restriction-based genotyping strategy (green and red squares).

A 60 bp oligonucleotide, containing our target sequence between a SP6 promoter region and a universal overlapping region was designed following Gagnon et al. Supplemental Protocols (ATTTAGGTGACACTATAGGTCGATCTTAAGTCCTTGGGTTTTAGAGCTAGAAATAGCAAG). The SP6 promoter is necessary for RNA transcription while the constant overlapping region is necessary for the annealing with the constant oligonucleotide

(AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGC TATTTCTAGCTCTAAAAC) that encodes for the final portion of the sgRNA, which is recognised and bind by the Cas9 enzyme. Annealing and sgRNA synthesis were performed following Gagnon et al. Supplemental Protocols.

Once synthetized the *stat3_ex14* sgRNA was stored at -80°C in small aliquots.

2.14.2 stat3_ex14 sgRNA injection and genotyping with HMA

stat3_ex14 sgRNA was injected into 1-2 cell stage WT embryos together with the Cas9 protein (M0386T, NEB). The injection mix contains 68 ng/uL of *stat3_ex14* sgRNA, 1X Danieu injection buffer, 1X Phenol red injection dye and 280 ng/uL Cas9 protein.

Mutagenesis efficiency was tested through the genotyping of a pool of 24 hours post fertilization injected embryos. Once extracted single embryo DNA was used for PCR amplification of the genomic portion containing *stat3* exon 14 sequence with the following primers:

- STAT3_ex14_FW: CCGAACCAATAGTGATGTAAGGC;
- STAT3_ex14_RV: TGAACTAATGAACACAACAAGCA.

PCR products were then analysed through Heteroduplex Mobility Assay: after denaturation at 95°C for 15' and slow renaturation, the PCR products were loaded on a 10% 29:1 polyacrylamide gel to look for additional heteroduplex bands with respect to the WT single band (Fig. 2.4).

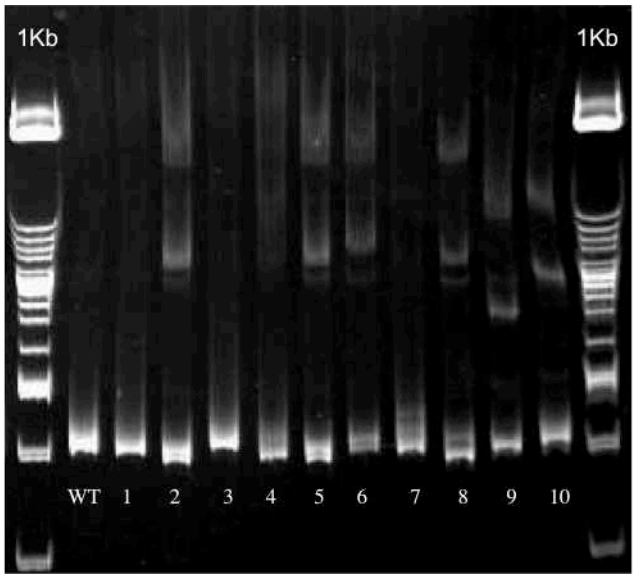

Figure 2.4 **Heteroduplex Mobility Assay.** HMA in 10% polyacrilamide 29:1 gel: WT PCR after denaturation/renaturation shows a single band migration profile which differs from samples 2, 5, 6, 8, 9, 10, thus indicating the presence of a different allele in the exon region amplified by PCR.

2.15 stat3Δ8bp mutant genotyping

A genotyping strategy was optimized by our Lab for detection of the *stat3Δ8bp* mutant allele, that possess an 8 bp deletion inside zebrafish *stat3* gene.

A canonical PCR amplification is performed using two primers flanking the deletion region that amplify a fragment of 164 bp (Fig. 2.5).

After amplification the PCR is loaded into a 4% agarose in TBE gel, in order to distinguish between mutant and WT allele: WT fish will display a single heavier band, homozygous fish will display a single lighter band while heterozygous fish will display both bands.

Primers sequences are the following:

- Stat3ex14short_FW: GGCCTCTCTGATAGTGACCG
- Stat3ex14short_RV: AGTTGTGCTTAGACGCGATC

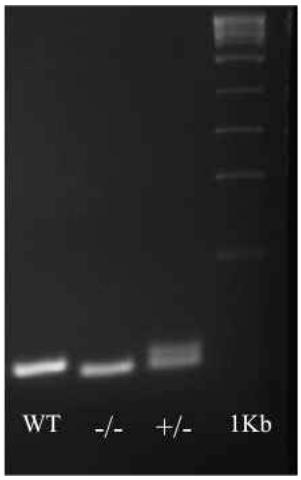

Figure 2.5 **Electrophoresis on 4% agarose gel for *stat3Δ8bp* allele detection.** WT DNA produces a single heavier PCR product , *stat3Δ8bp-/-* DNA gives a single lighter PCR band while heterozygous DNA is characterized by the presence of both PCR products.

2.16 EdU proliferation assay

EdU proliferation assay was performed using the Click-iT® EdU Alexa Fluor® 594 Imaging Kit (Thermo Fisher Scientific) following manufacturers instruction.

After initial exposure to EdU reagent, *Tg(7xStat3-Hsv.Ul23:EGFP)* 48 hpf embryos and *Tg(7xStat3-Hsv.Ul23:EGFP)* 4 dpf larvae were washed to eliminate residual EdU and kept in fish water for respectively 1h 30' and 24 h to allow compound intercalation with DNA of mitotic cells. Proliferating cells were later on stained with Alexa 594 red fluorophore and fixed in 4% PFA in PBS O/N at 4° for fluorescence *in situ* hybridization or immunofluorescence staining.

2.17 P-H3 proliferation assay

The mitotic rate of embryos and larvae after chemical treatments or mRNAs injection was visualized in a tissue specific manner through the whole mount P-Histone 3 proliferation assay. The protocol was optimized by our Lab: samples stored in methanol were re-hydrated with progressive MetOH to PBS washes and after re-equilibration with one wash in ddH2O, permeabilization was performed in pure Acetone for 7' at -20°C indiscriminately on the age of the sample. Samples were then blocked in PBDT (2 mg/ml BSA, 1% DMSO, 0,5% Triton X in PBS) + 2% sheep serum for 1 hour at room temperature and incubated O/N at 4°C in rabbit anti-P-H3 1° antibody (1:1000 in PBDT/2%sheep serum). After removal of the excess of 1°antibody with PBDT washes, the secondary anti-rabbit-AP antibody was incubated O/N at 4°C (1:1000 in PBDT). Antibody detection was performed with NBT/BCIP solution, a substrate for alkaline phosphatase enzyme.

2.18 Image acquisition and analysis

Fluorescence was visualized and acquired using the conventional fluorescence dissecting microscope Leica M165FC and the confocal microscope Nikon C2 H600L. For brightfield and fluorescence imaging with the dissecting microscope, embryos and larvae were anesthetized with tricaine and mounted in 2% methylcellulose. For confocal imaging, anesthetized embryos and larvae and anatomical samples of adult fish were embedded in

1% low melting agarose. The fluorescence was visualized using 488 nm (for EGFP) and 561 nm (for RFP) lasers and 20x or 40x immersion objectives (Nikon). Sections of adult organs were visualized with a 100x oil immersion objective (Nikon).

Fluorescence quantification of the images acquired either with the conventional fluorescence or the confocal microscope was carried out using Fiji software. Each image, saved as TIFF file and visualized as a RGB colour image, was splitted in the three colour components and only the channel corresponding to the fluorophore of interest was kept (red for RFP and green for EGFP). A threshold was set to have a good signal-to-noise ratio and maintained for all the images of the experiment. Fluorescence was quantified as integrated density. For fluorescence quantification of whole embryos in images acquired at the conventional fluorescence microscope, the yolk and yolk extension areas were excluded from the analysis. Statistical analyses were carried out with Excel software.

2.19 DNA extraction

DNA extraction was performed following the HotSHOT protocol by Hutchinson et al., (2007) that works for embryos, larvae and adult tissue samples.

Once removed the fishwater, the sample is treated with 50mM NaOH at 95°C and then the basic solution is neutralized by addition of 1/10 of the volume of 1M Tris HCl pH 7.5.

For single embryos or larvae 20 ul NaOH were used and incubation was performed for 10’ at 95°C. Fin tails were incubated in 100uL NaOH at 95°C for 20’.

Finally the samples were centrifuged for 3’ at 16000 rpm and 2uL DNA solution was used for 20uL PCR volume.

3. RESULTS

3.1 Generation and validation of a Stat3 zebrafish reporter line

3.1.1 Generation of C-reactive protein-based Stat3 reporter construct

A zebrafish Stat3 reporter is a transgenic biosensor fish that expresses a fluorescent protein under the control of regulatory sequences responsive to Stat3 signaling pathway. The Stat3 responsive elements (SRE) are specific sequences in the promoter of Stat3 target genes, which are recognised and bound by Stat3 transcription factor. We obtained the SRE that were further used to design the reporter construct, from the pLucTKS3, a plasmid that contains seven copies of an oligonucleotide (TTCCCGAA), corresponding to a Stat3-specific binding site from the human C-reactive protein (CRP) gene (nucleotides -123 to -85) (Turkson et al., 1997). The pLucTKS3 also contains as minimal promoter an oligonucleotide corresponding to the -35 to +11 region (relative to the transcriptional start site at +1) of the herpes simplex virus thymidine kinase (HSV-TK) promoter (Turkson et al., 1997). Although the sequences of the natural palindromic STAT3-binding elements vary considerably in both human and zebrafish promoters, they conform to the general structure TT$(N)_4$AA (Seidel et al., 1995). The specificity of the chimeric pLucTKS3 reporter had already been demonstrated *in vitro* through Luciferase activity assays, that proved the exclusive activation via Stat3 protein (Turkson et al., 1997).

With the aim to generate a Stat3 reporter construct suitable for injection in the zebrafish embryo, the previously described TKS3 fragment from the pLucTKS3 reporter plasmid was used to create the recombinant plasmid pDestTg(*7xStat3-Hsv.Ul23:EGFP*)_A2 following the Tol2-based Gateway recombination reaction. To this aim the TKS3 promoter fragment was first cloned into a Gateway 5' entry clone (p5E-MCS) to generate the p5E-TKS3 vector. This p5E-TKS3 was subsequently recombined together with a middle entry clone, containing the EGFP reporter gene, and a 3' entry clone, containing the SV40 late polyA signal (p3E-polyA), in the Tol2 transposon backbone pDestTol2pA2, through a MultiSite Gateway LR recombination reaction.

The Gateway destination vector we obtained was named pDestTg(*7xStat3-Hsv.Ul23:EGFP*)_A2 (Fig. 3.1) and contains, flanked by Tol2 recombination sites, the transgenic cassette that drives EGFP expression under the control of the TKS3 promoter.

These Tol2 sites in the presence of a transposase enzyme will allow random insertion of the reporter construct into the genome of the injected embryos. Hence, one cell stage WT embryos were injected with a mix containing the pDest*Tg(7xStat3-Hsv.Ul23:EGFP)_A2*, together with a transposase mRNA, and the mosaic transgenic fish showing a partial fluorescence pattern at 24 hpf, were raised to adulthood and outcrossed. The F1 progeny was screened for fluorescent fish, and the identified F0 founders were used to establish the stable *Tg(7xStat3-Hsv.Ul23:EGFP)* reporter line.

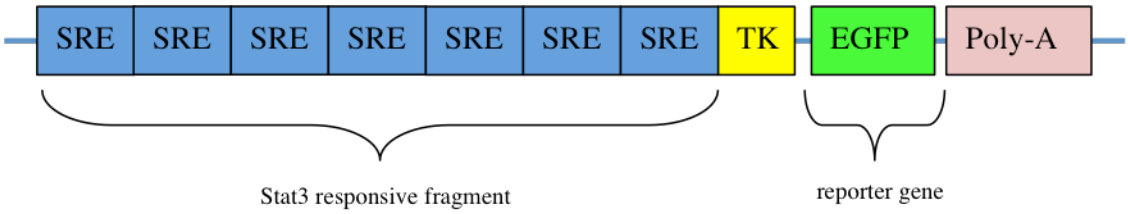

Figure 3.1 Schematic representation of the CRP-based Stat3 reporter transgene in the Gateway destination vector. The human CRP promoter fragment (Stat3 REs and TK minimal promoter) controls the expression of the EGFP reporter protein.

3.1.2 *Tg(7xStat3-Hsv.Ul23:EGFP) transgenic line is a bona fide Stat3 reporter*

The reliability of the promoter we used to generate the transgenic line had already been confirmed *in vitro (Turkson et al., 1998)*. However, we needed to prove the *in vivo* responsiveness of the novel *Tg(7xStat3-Hsv.Ul23:EGFP)* transgenic line we generated. A valid reporter fish should in fact be able to return proportional variations in the detectable signal, to the activity of the pathway under investigation (Moro et al., 2013). Perturbations to the pathway activity are thus necessary to prove the reporter to be a reliable tool for *in vivo* investigations. The major approaches adopted to validate a zebrafish reporter line are represented by application of genetic or pharmacological interferences (Moro et al., 2013).

3.1.2.1 Pharmacological inhibition of Stat3 signaling pathway decreases Tg(7xStat3-Hsv.Ul23:EGFP)reporter signal

To achieve a chemically-induced inhibition of the signaling pathway, we selected two well known Stat3 inhibitory drugs, AG490 and LLL12 and administrated them to *Tg(7xStat3-Hsv.Ul23:EGFP)* reporter embryos. AG490 acts by blocking the JAK2 Kinase, responsible to the post-translational phosphorylation of Stat3 Tyr708 (Huang et al., 2010), which is necessary for dimerization preceding nuclear migration of the transcription factor. LLL12 molecule binds directly the Stat3 SH2 domain, where the Tyr708 is located, creating steric interference and thus preventing phosphorylation by the JAK2 Kinase (Lin et al., 2010) (Fig. 3.2 B).

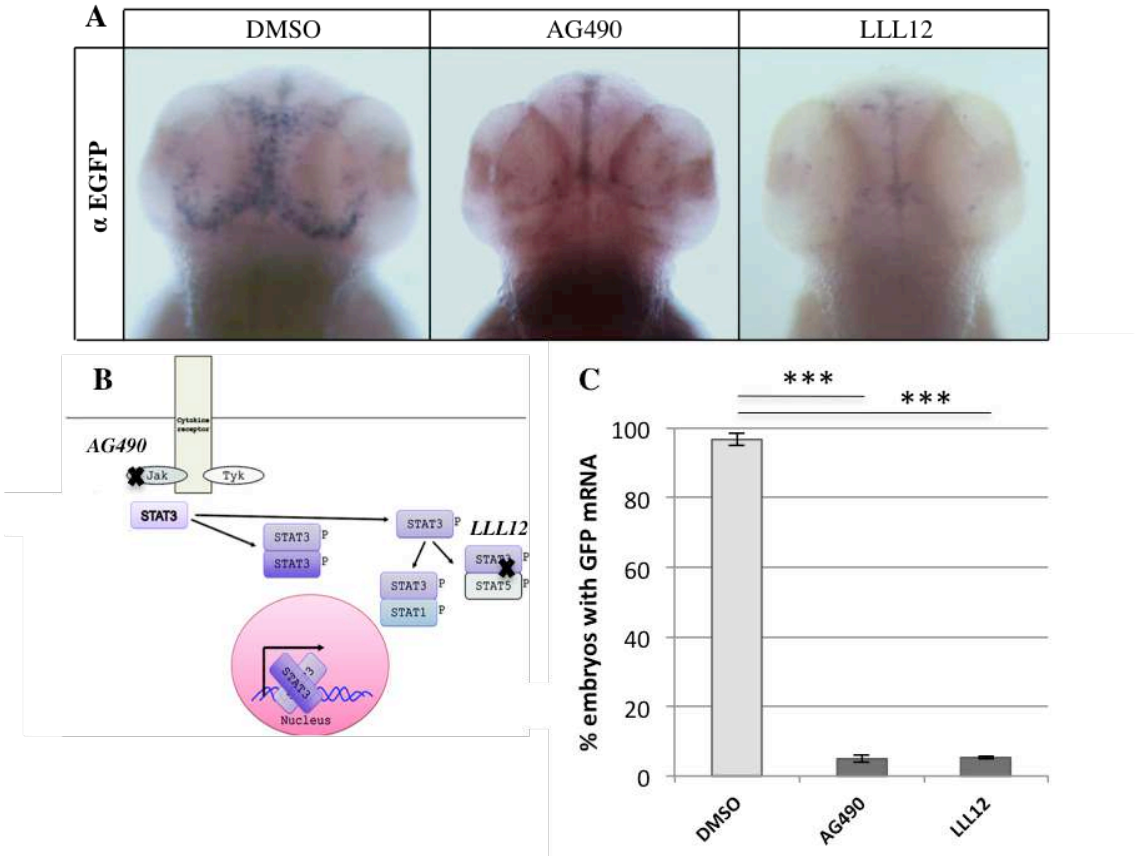

Figure 3.2 Stat3 inhibitory drugs significantly reduce EGFP expression in the *Tg(7xStat3-Hsv.Ul23:EGFP)*reporter embryo. EGFP mRNA expression in the optic tectum is abolished at 48hpf in both AG490 and LLL12 treated embryos with respect to the DMSO control (A). AG490 and LLL12 significantly reduce the number of transgenic embryos that express EGFP reporter mRNA (C). Schematic representation of the molecular mechanism of Stat3 pathway inhibition by AG490 and LLL12 (B).

The drugs were administrated to 24 hpf embryos by dissolving the compound into the embryo medium, and fluorescence was checked after 24 hours treatment. Because of the strong green auto fluorescence emitted by the drugs solutions, EGFP expression was detected by ISH with an anti-EGFP probe. Both treatments abolished EGFP gene expression with respect to the untreated controls, confirming that chemical perturbations to the Stat3 pathway can be reliably reported by the *Tg(7xStat3-Hsv.Ul23:EGFP)* embryo (Fig. 3.2).

3.1.2.2 *Exogenous stimulation of JAK/Stat3 pathway is reporter by Tg(7xStat3-Hsv.Ul23:EGFP) EGFP production*

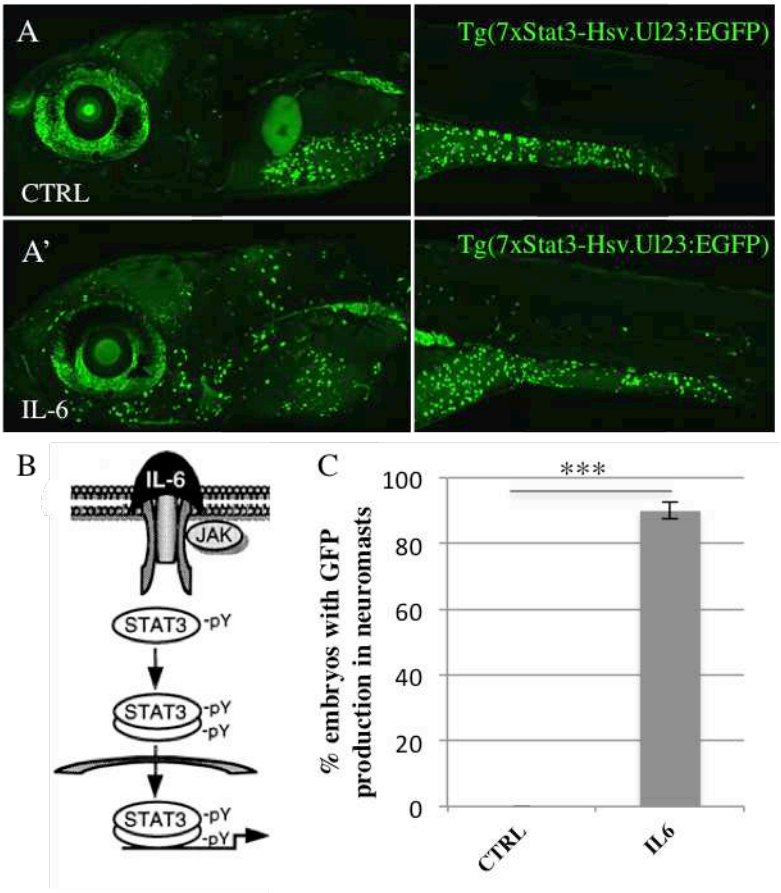

Figure 3.3 IL-6 activates *Tg(7xStat3-Hsv.Ul23:EGFP)*reporter signal in the neuromasts of the head and the trunk. IL-6 human recombinant protein injection stimulated EGFP production in the neuromasts of the head and the trunk of *Tg(7xStat3-Hsv.Ul23:EGFP)*larvae (A,A',C). Schematic representation of IL-6 activation mechanism of Stat3 pathway (B).

To induce an exogenous activation of Stat3 signaling we chose IL-6 human recombinant protein that, despite low sequence homology, shows high structural similarity with zebrafish IL-6 (Varela et al., 2011). IL-6 acts as a positive regulator of STAT3 by binding its specific transmembrane receptors IL-6R in the outer membrane, and thus giving rise to the molecular cascade that takes to Stat3 Tyr708 phosphorylation and transcription activation of STAT3 target genes (Fig. 3.3 B). The protein was injected directly into the heart of 48 hpf embryos, and the result was observed at 7 dpf through confocal imaging using the Nikon C2 confocal microscope. Coherently, *Tg(7xStat3-Hsv.Ul23:EGFP)* reporter showed non-physiologic EGFP production in those tissues where IL-6R presence has been documented during the first week of larval development: the head, the epidermis and the neuromasts of the anterior and posterior lateral line system (Varela et al., 2011) (Fig. 3.3 A,A'). Significantly, 87% of the injected embryos showed non-physiologic EGFP production with respect to 0% of the controls (Fig. 3.3 C).

3.1.2.3 Genetic activation of Stat3 signaling pathway increases Tg(7xStat3-Hsv.Ul23:EGFP) reporter signal

Two different mRNAs encoding for the murine WT and constitutively active form of Stat3 (Stat3C) were injected to obtain an over-activation of the pathway in *Tg(7xStat3-Hsv.Ul23:EGFP)* embryos. The constitutive activity of murine Stat3C, caused by the two modifications A661C and N663C, is dependent on a spontaneous dimerization, directed by di-sulphide bonds, that increases Stat3C DNA-binding activity, resulting in a faster on-rate and slower off-rate. Notably, tyrosine phosphorylation upon cytokine or mitogen-induced signaling events is obligatory for Stat3C activation (Li and Shaw, 2006).

mRNA injection was performed at 1 cell stage and a strong EGFP expression was detectable by confocal analysis from 5 dpf, specifically in the head and the trunk of the larvae. The timing and the EGFP expression pattern after injection of the either Stat3 or Stat3C mRNAs was very similar, coherently with the dependence on an upstream activation signal; however, Stat3C injected *Tg(7xStat3-Hsv.Ul23:EGFP)* larvae showed much stronger fluorescence intensity with respect to the same batch of larvae injected with the WT form of Stat3 (Fig. 3.4). Together these data clearly indicate the

competence of the *Tg(7xStat3-Hsv.Ul23:EGFP)* transgenic line to report *in vivo* an increase in Stat3 pathway activity.

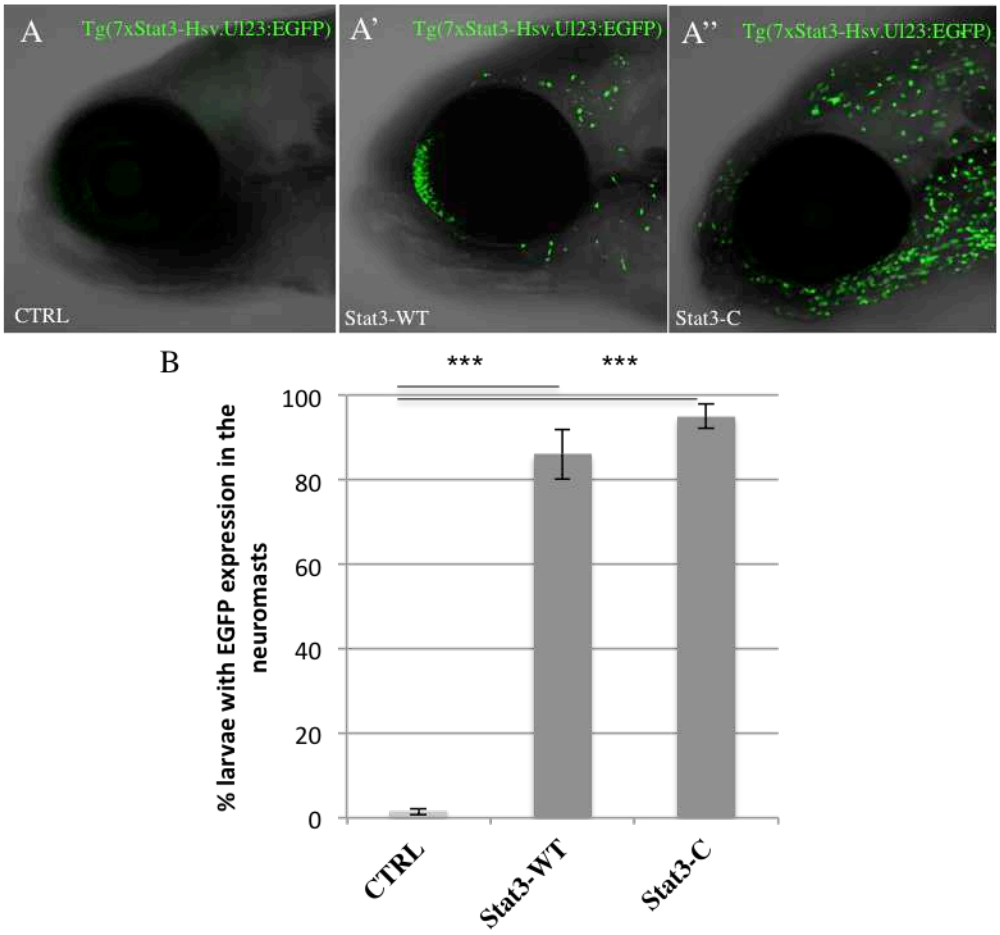

Figure 3.4 WT and constitutively active forms of murine Stat3 increase *Tg(7xStat3-Hsv.Ul23:EGFP)*fluorescence. Injection of both Stat3-WT and Stat3-C isoforms cause a significant increase of the *Tg(7xStat3-Hsv.Ul23:EGFP)* reporter fluorescence, in the head neuromasts of 5 dpf larvae (A,A',A'',B).

3.2 *In vivo* characterization of Stat3 expression in zebrafish

3.2.1 *Stat3 reporter expression is activated maternally*

Tg(7xStat3-Hsv.Ul23:EGFP) fluorescence is detectable in the embryo already at 1 cell stage when fish derive from transgenic females crossed to wild-type fish. However, no signal is observed at this same stage in the offspring of male carriers (Fig. 3.5). This result suggests that Stat3 target genes mRNAs and proteins are inherited maternally from the oocyte, since EGFP expression is detectable before the Midblastula Transition (MBT), that occurs normally at 512 cell stage (2.75 hpf) and represents the moment when zygotic transcription starts. Before the MBT all mRNAs and proteins that are present in the developing embryo are maternal products deposited in the egg during oogenesis.

Thus, the presence of EGFP expression shortly after fertilization is only ascribable to Stat3 pathway activation during oogenesis.

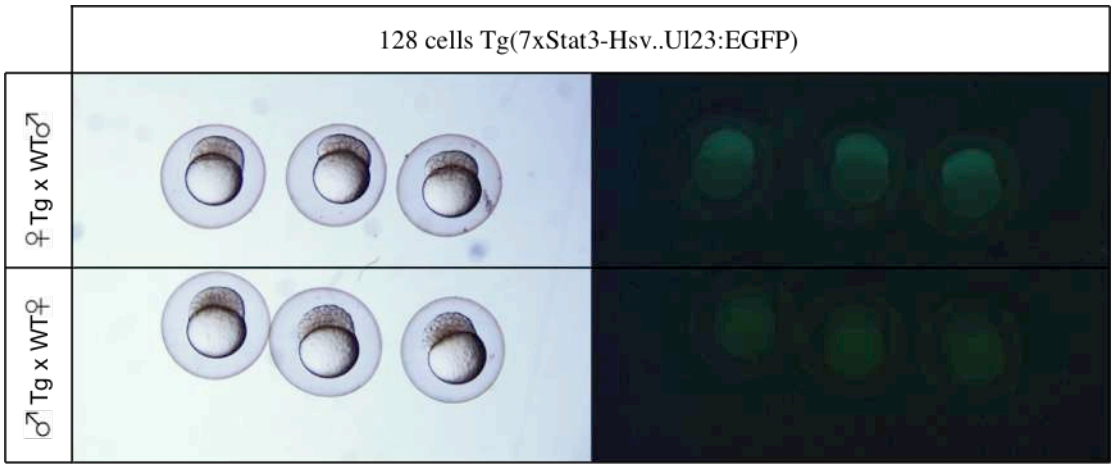

Figure 3.5 Stat3 target genes are maternally inherited by the oocyte. EGFP expression reporting Stat3 pathway activation is present shortly after fertilization only in the embryos derived from transgenic females outcrossed with WT males.

3.2.2 Stat3 pathway is active in the nervous system and in the gut during development

Stat3 pathway activation occurs at 20 hpf during late somitogenesis. At this stage the reporter signal is stronger in the anterior telencephalic region, where the formation of the anterior intra-encephalic sulcus (AIS) is taking place (Fig.3.6 A,B). These cell movements will define the so called everted telencephalic structure, which is typical of Ray-finned fish. More dorsally two defined stripes of cells are marked in the developing forebrain, and in the primordium of the Midbrain Hindbrain Boundary (MHB). Fig. 3.6 C shows the strong activation of the reporter activity in those cells that form the sensory cranial ganglia of the zebrafish, known to develop between 20 and 25 somite stages. Finally, in the superficial epithelium of the head and the trunk of 22 hpf *Tg(7xStat3-Hsv.Ul23:EGFP)*embryos, the primordia of the anterior and posterior lateral line are labelled (Fig. 3.6 D). These cells will originate the neuromasts, small sensory patches located superficially on the skin or just under the skin in fluid-filled canals on the head and along the trunk of the zebrafish larvae (ZFIN).

In the 48 hpf *Tg(7xStat3-Hsv.Ul23:EGFP)* reporter, the fluorescence is mostly located in the midbrain, where the optic tectum (OT) cells are rapidly proliferating and differentiating (Fig. 3.6 E,F). The radial growth of proliferating cells that creates a cell migration movement from the peripheral region of the OT to the center of each lobe, is followed by a differentiation gradient (Devès and Bourrat, 2012); EGFP expression in the OT seems to be inversely proportional to this gradient, resulting more detectable in the peripheral region and then progressively fading at the center of the lobe. In addition, at 48 hpf a low number of single cells is marked by EGFP in the inner retina, another highly proliferating tissue at this stage of development (Fig. 3.6 E).

After 48 hpf the reporter signal disappears from the nervous system, to appear in the developing gut starting from 4 dpf (Fig. 3.6 G). At this stage a strong salt and pepper fluorescence starts to be visible in the single layer of cells that surrounds the newly forming intestinal lumen (Fig. 3.6 H). Moreover, confocal analysis on EGFP expressing cells revealed a distinctive pear-shaped morphology (Fig. 3.6 I).

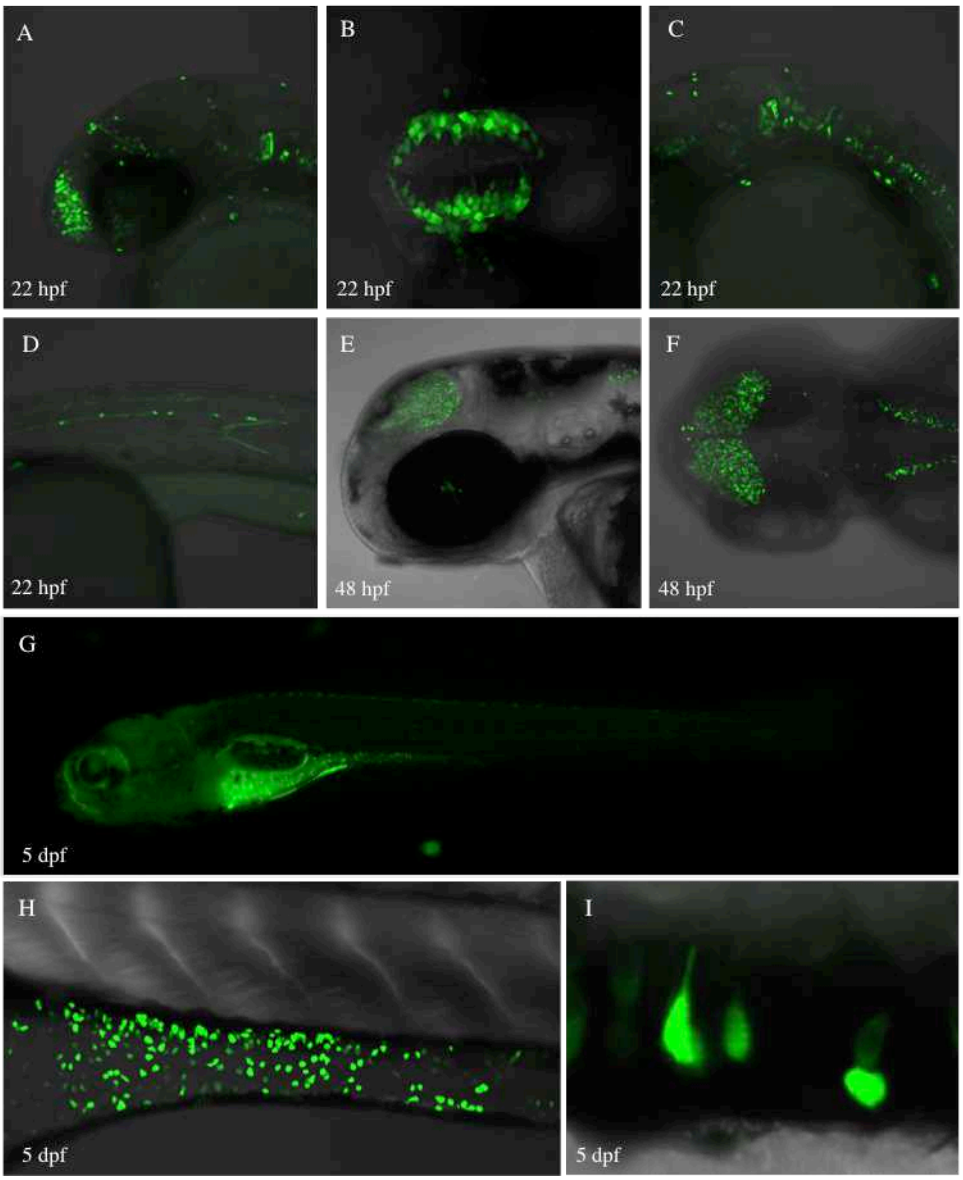

Figure 3.6 ***In vivo*** **expression pattern of** ***Tg(7xStat3-Hsv.Ul23:EGFP)*** **reporter from 24 hpf to 5 dpf.** Stat3 pathway is active in the telencephalon, the midbrain/hindbrain boundary and the hindbrain at 22 hpf (A,B,C). Reporter signal also marks the neuromast progenitor cells along the lateral line of 22 hpf embryos (D). At 48 hpf EGFP reporter signal is located in the optic tectum and the cerebellum (E,F). Starting from 4 dpf Stat3 signaling pathway is present in the developing intestine, with a single cell activation pattern (H) while it fades in the nervous system (G). Intestinal Stat3 positive cells possess a distinguishable triangular shape (I).

3.2.3 *Tg(7xStat3-Hsv.Ul23:EGFP) reporter signal is active at the base of adult intestinal inter-villus pocket*

Stat3 reporter gene expression in the developing gastro-urinary tract of zebrafish model, starts to be detectable at 4 dpf. EGFP production is from that moment persistent in the intestinal tissue throughout adulthood, with the same "salt and pepper" pattern observed in the earlier larval stages.

Danio rerio intestinal mucosa does not possess proper crypts, nevertheless the inter-villus pocket has to date been considered a valid model for the investigation of the molecular mechanisms connected with cell differentiation. The histology and the cell type distribution along the intestinal villus resemble those of mammal and human models, with a positive differentiation gradient moving from the base to the top of the anatomical structure (Devès and Bourrat, 2012). Cells that occupy the lower portion of the crypt are thus undifferentiated stem cells defined Crypt Base Columnar cells (CBC) or highly proliferative Transient Amplifying cells (TA) (Fuji and Sato, 2014) (Fig. 3.7 B); while moving up to the top of the villus the cells receive fate-determining molecular inputs, thus becoming either absorptive Enterocytes, or mucosal Goblet Cells, or hormones secreting Enteroendocrine Cells (Ng et al.,2005). Remarkably, zebrafish intestine does not possess Paneth like cells (Ng et al.,2005).

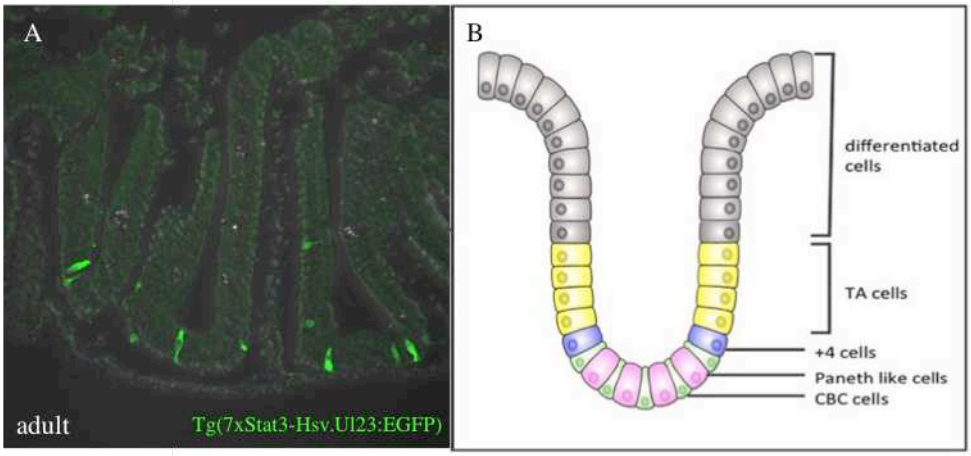

Figure 3.7 Stat3 signaling pathway is active at the base of the adult intestinal inter-villus pocket. IF staining using α-EGFP antibody marks single cells in the basal portion of the inter-villus pocket of the adult *Tg(7xStat3-Hsv.Ul23:EGFP)* intestine (A). Schematic representation of the actual cell-type distribution model for mammals intestine from Fuji and Sato, 2014 (B).

Stat3 target cells were identified by IF using anti-GFP Ab on adult *Tg(7xStat3-Hsv.Ul23:EGFP)* intestine sections. EGFP producing cells are only localized at the base of the inter-villus pocket, thus revealing that Stat3 is only expressed by a small number of undifferentiated cells in the adult zebrafish intestine (Fig. 3.7 A).

3.2.4 Stat3 marks proliferating cells during zebrafish development

Even though many evidences have been collected to date to demonstrate Stat3 involvement in the regulation of proliferation, most of them were observed in the pathological context of a neoplastic transformation. Stat3 was in fact found over-expressed in nearly 70% of human solid and haematological tumours, where its ectopic transcriptional activity was associated to tumor formation, invasion and metastasis (Yu et al., 2014).

In mouse, Stat3 depletion leads to embryonic lethality, assessing its importance during early development (Takeda et al., 1997); none the less, little is known about its physiological function during tissues development.

The *Tg(7xStat3-Hsv.Ul23:EGFP)* reporter gave us the possibility to follow *in vivo* the spatiotemporal activation of the Stat3 pathway in different tissues with single cell resolution.

The EGFP expression pattern was mainly localized in those tissues that during zebrafish developmental stages are known to be characterized by high cell division rate: the telencephalon, the optic tectum and the inner retina are tissues that express high levels of proliferation markers during the first days of embryonic development (Dèves and Bourrat, 2012). Moreover, EGFP mRNA expression in the gut, starts to be detectable with ISH from 3 dpf (data not shown), when cell proliferation in the primitive gut has been documented to begin (Ng et al., 2005). Finally, the localization of Stat3 positive cells at the base of the zebrafish intestinal crypt brought the curiosity to investigate the cells division rate in *Tg(7xStat3-Hsv.Ul23:EGFP)* fluorescent cells.

At 48 hpf mitotic cells were stained performing 5-ethynyl-2'-deoxyuridine (EdU) Proliferation Assay, thus allowing the labelling of dividing cells in whole mount zebrafish embryos or larvae. EdU is efficiently incorporated into DNA during replication, thus labels dividing cells within the selected period of interest. Compound incorporation is detected by an Alexa Fluor 555 fluorophore. The procedure was at this stage coupled

with fluorescent ISH using anti-EGFP probe, stained with Fast Red fluorophore. A Leica SP5 confocal then isolated the two different fluorescent signals that were found to co-localize in the stem/progenitor cell zone of the optic tectum (Fig. 3.8 A-A'').

The same EdU proliferation assay was then used to label 5 dpf intestinal proliferating cells, while EGFP expressing cells where identified by IF staining (Fig. 3.8 B-B'').

Notably, co-localization analysis showed that each cell that incorporated EdU during the selected period of time, is also EGFP positive.

Taken together these data indicate that Stat3-dependent DNA transcription is active during the first days of zebrafish development, in mitotic cells of the nervous system and gastrointestinal tract.

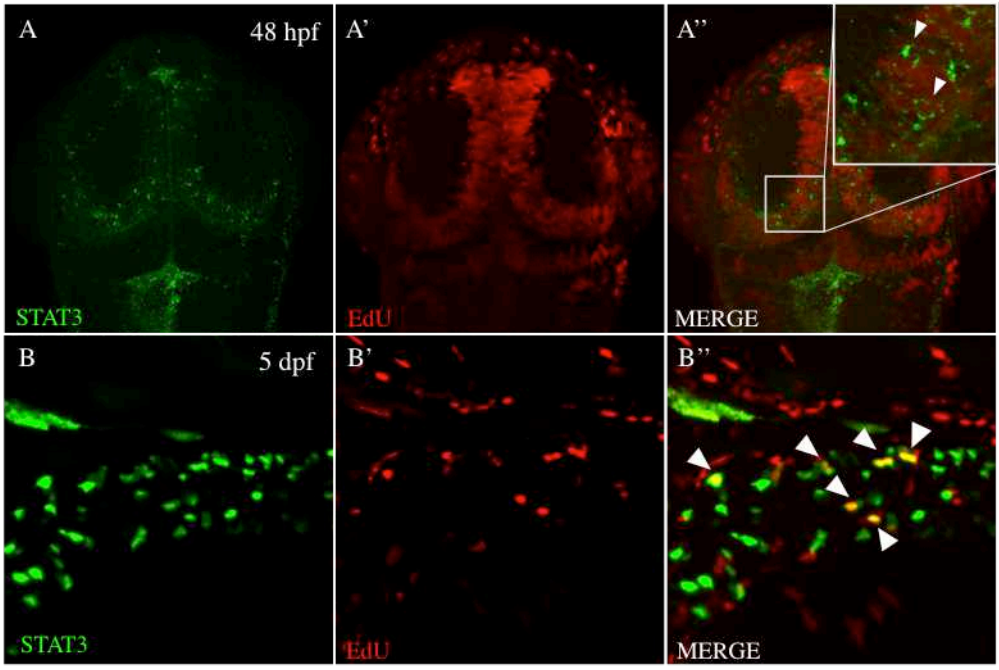

Figure 3.8 Stat3 marks proliferating cells in the nervous system and the intestine during zebrafish development. FISH using α-EGFP probe (A) and Edu proliferation assay stained with Alexa 594 red fluorophore (A') co-localize in the optic tectum of 48 hpf *Tg(7xStat3-Hsv.Ul23:EGFP)* reporter embryos (A''). IF staining using α-EGFP Ab (B) and Edu proliferation assay (B') co-localize (arrowheads) in the developing intestine of 5 dpf Stat3 reporter larvae (B'').

A second evidence confirming Stat3 activity in proliferating cells was obtained through an *in vivo* Label Retention Assay, using the *Tg(HSP70:H2B:mRFP)* transgenic line, that marks cell nuclei with a red fluorophore after a heat shock pulse .

Cells accumulate RFP indiscriminately already six hours after the heat induction, and afterwards the fluorophore is diluted proportionally to the proliferation rate of the cell (Fig. 3.9). By crossing *Tg(7xStat3-Hsv.Ul23:EGFP)* and *Tg(HSP70:H2B:mRFP)* transgenic lines, we were able to follow the temporal loss of RFP in the intestinal Stat3 expressing cells, for the 48 hours *post* heat shock (hpHS) in 6 dpf larvae. Importantly, already 24 hpHS the percentage of Stat3 positive RFP retaining cells was significantly reduced to 15%, with respect of the 100% labelled cells at 6 hpHS, while at 48 hpHS none of the observed samples showed any co-localization between EGFP and RFP (Fig. 3.9 B). This remarkable result demonstrates that Stat3 expression is only present in those cells that rapidly undergo mitosis in the young zebrafish intestine.

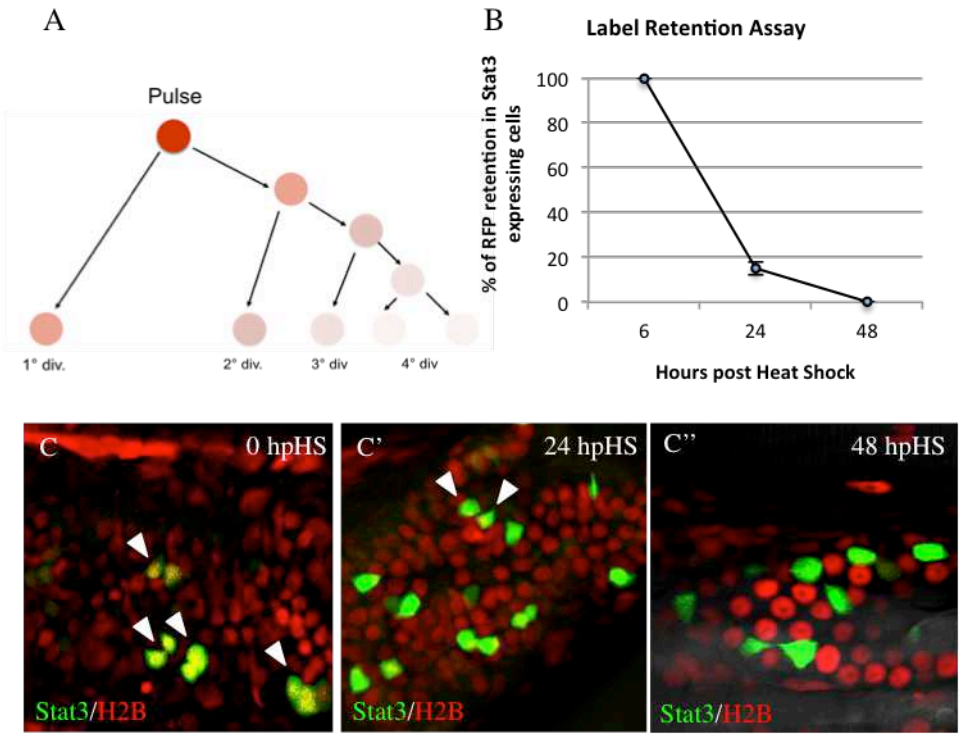

Figure 3.9 Stat3 target genes are expressed in actively proliferating intestinal cells at 6 dpf. Schematic representation of a Label Retention Assay using a red fluorophore; each mitosis dilutes the fluorescence intensity (A). Double transgenic *Tg(7xStat3-Hsv.Ul23:EGFP)/Tg(HSP70:H2B:mRFP)* 5 dpf larvae showed accumulation of RFP label at 6 hpHS, ubiquitously in the intestine (C); at 24 hpHS less than 20% of Stat3 expressing cells were retaining the RFP (arrowheads) (C'), while at 48 hpf no more EGFP/RFP co-localization was appreciable (C''). Graphic representation of RFP time-dependent loss by Stat3 positive intestinal cells (B).

3.2.4.1 Depletion of post-mitotic signals causes Stat3 pathway activation

The cell cycle consists of four distinct phases: G1 (Gap1) phase, S phase (synthesis), G2 (Gap2) phase (collectively known as interphase) and M phase (mitosis). Cells that have temporarily or reversibly stopped dividing are said to have entered a state of quiescence called G0 phase. G0 is sometimes referred to as a "post-mitotic" state, since cells in G0 are in a non-dividing phase outside of the cell cycle. Previous studies demonstrated TGF-β signaling pathway to exert a post mitotic signal in zebrafish neuronal precursors (Casari et al, 2014), determining a Smad3 mediated cell cycle exit. An important issue to definitely correlate Stat3 transcriptional targets expression and proliferation was achieved upon chemical inhibition of the TGF-β pathway.

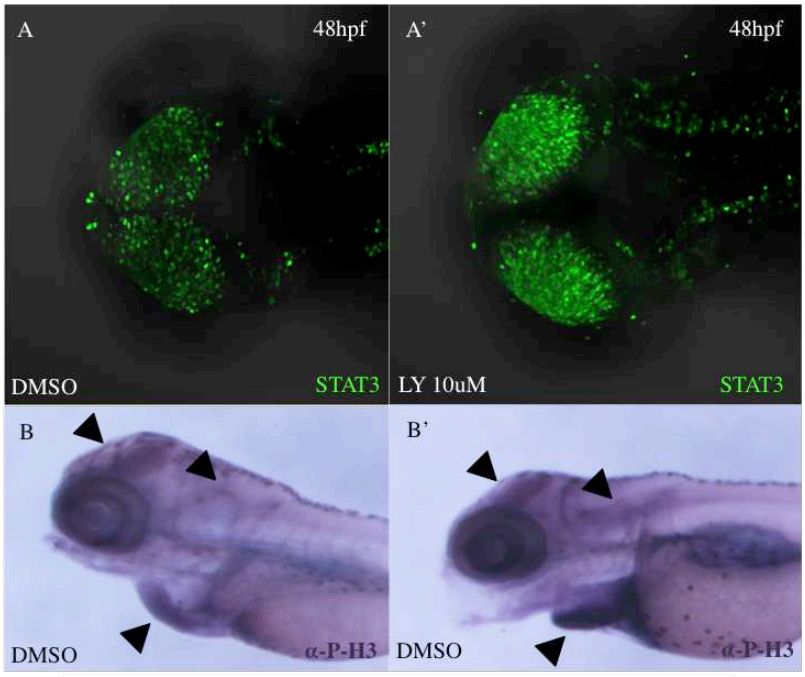

Figura 3.10 **Depletion of post-mitotic signals increases Stat3 signaling pathway in the optic tectum of 48 hpf embryos.** *in vivo* fluorescence reporting Stat3 signaling activity is increased in the optic tectum of *Tg(7xStat3-Hsv.Ul23:EGFP)* embryos treated with LY inhibitor (A') with respect to untreated controls (A). α-PH3 proliferation assay revealed an increase of the mitotic rate subsequently to LY treatment (arrowheads)(B') with respect to the untreated control (B). Relative fluorescence measured in the optic tectum of 48 hpf *Tg(7xStat3-Hsv.Ul23:EGFP)* embryos is significantly increased upon LY treatment (C).

24 hpf *Tg(7xStat3-Hsv.Ul23:EGFP)* embryos were treated with the known LY364947 Alk4-5/Smad3 receptor kinase inhibitor, thus blocking the post-mitotic signal responsible of the cell cycle arrest in the developing neural system.

Consistently with our expectations, increased mitotic index in the optic tectum of treated embryos, which was assessed by a pH3 IHC, came together with a significant over-expression of the EGFP reporter signal in treated *Tg(7xStat3-Hsv.Ul23:EGFP)* embryos with respect to the DMSO treated controls (Fig. 3.10). Stat3 signaling pathway activation is then proportional to the number of mitotic cells in the zebrafish developing optic tectum.

3.2.5 Stat3 is expressed by Crypt Base Columnar Cells in zebrafish adult intestine

The novel *Tg(7xStat3-Hsv.Ul23:EGFP)* reporter was used in previous experiments to verify the hypothesis that Stat3 signaling pathway is active in mitotic cells during zebrafish development. Notably, Stat3 activity reported in the *Tg(7xStat3-Hsv.Ul23:EGFP)* fish, was observed to persist in the adult intestine, maintaining an isolated cells pattern. Moreover, EGFP signal in adult fish intestinal sections was always detected at the base of the inter-villus pocket, where, specifically for zebrafish, cells are maintained in an undifferentiated proliferating state. In mammals, the population of basal crypt cells is composed of three cell types in the large intestine: Crypt Base Columnar cells (CBC), Paneth cells and +4 cells. CBC are Lgr5+ intestinal stem cells that divide every 24 h, giving rise to progeny called "transit-amplifying cells" (TA cells) that reside just above the crypt stem cell zone (Fujii and Sato, 2014). The Paneth cells are differentiated lysozyme secretory cells, while +4 cells are quiescent cells that may upon damage revert back to Lgr5+ cells, thus acting as ISC reservoir (Tian et al., 2011). Notably, in mammals, the small intestine lacks both villi and Paneth cells, thus being more similar to zebrafish intestine histology.

With the aim to distinguish the specific Stat3 expressing cell type, we performed a double IF staining with both EGFP and PCNA antibodies on adult *Tg(7xStat3-Hsv.Ul23:EGFP)* intestinal sections. Results reported in Fig. 3.11 (A-A') show that each Stat3 reporter cell is proliferating in the zebrafish intestinal crypt. Afterwards, to distinguish between TA and CBC cells, we performed an Immunogold labelling with an anti-GFP Ab, that is then localized on the sample by a secondary Ab conjugated with a gold 10nm sphere. Adult *Tg(7xStat3-Hsv.Ul23:EGFP)* intestinal sections were then observed by Transmission Electron Microscopy, to detect gold-labelled cells. Strikingly, gold spheres were significantly more abundant in the cytoplasm of cells at the base of the intestinal folds, with respect to those located upstream the intestinal protrusions. Moreover, EGFP Ab selectively stained cells with a triangular shape and a big dense nucleus that occupies the most of the cell area and that is delocalized at the bottom part of the cell body: these are distinctive features of CBC cells (Fig. 3.11 B-B'').

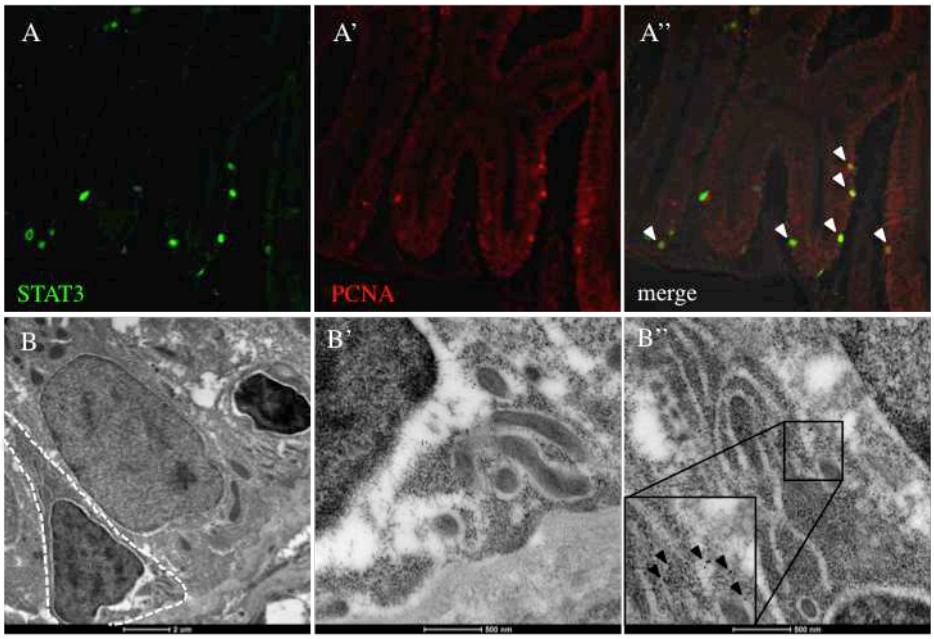

Figure 3.11 Stat3 marks proliferating Crypt Base Columnar cells in the adult zebrafish intestine. Double IF staining with α-EGFP (A) and α-PCNA (A') Abs reveals Stat3 as a marker of proliferation in the adult zebrafish intestine through perfect co-localization (A''). Immunogold with chicken α-EGFP 1° Ab and α-chicken-gold conjugated 2°Ab on zebrafish adult intestinal sections observed by TEM; gold-labelled cell is surrounded by white striped line (B); high magnification of gold-labelled cytoplasm belonging to triangular shaped crypt cell (B', B'') and zoom on gold dots (B'').

3.3 Stat3 regulates proliferation in the intestine of developing larvae

Previous experiments confirmed our hypothesis, that Stat3 signaling pathway is active in mitotic cells during zebrafish gut development, and that Stat3 is a marker of stemness in the adult intestinal crypt. At this point our interest was to answer the question: Is Stat3 itself regulating proliferation or is it activated downstream of a mitotic signal?

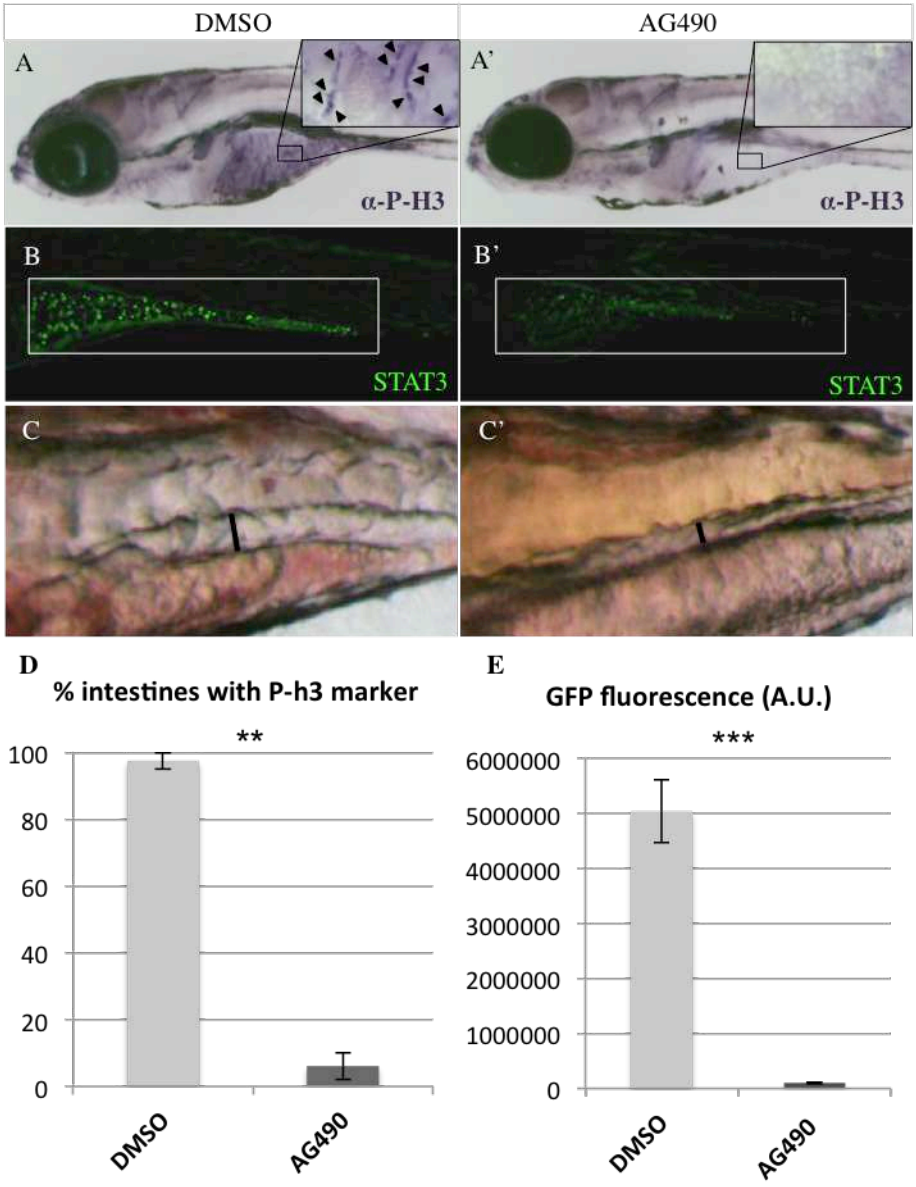

Figure 3.12 **Mitosis in the developing intestine of zebrafish larvae depends on Stat3.** αP-H3 proliferation assay on 6 dpf larvae (A,A'); AG490 treatment causes reduction of intestinal proliferation (A') if compared to the DMSO control (A). Stat3 reporter signal is significantly reduced in the intestine of AG490 treated larvae (B') with respect to the DMSO control (B). The thickness of zebrafish intestinal mucosa is notably decreased by AG490 Stat3 inhibitor (C,C'). Graphic representation of αP-H3 proliferation assay upon AG490 treatment (D). Graphic representing EGFP relative fluorescence measurements in the intestine of AG490 and DMSO treatments (E).

We then decided to inhibit Stat3 pathway at 3 dpf through the known AG490 Jak2 inhibitor, and observe the intestinal proliferation rate after 3 days of treatment, performing a αP-H3 assay. The timing of drug exposure was chosen between 3 and 6 dpf because during this period, the initial and more relevant steps of larval intestinal formation occur. 6 dpf treated larvae showed a significant reduction in the intestinal proliferation marker, consistent with the decrease in the Stat3 reporter signal (Fig. 3.12 A-B'). Interestingly, a more detailed microscopic analysis of the intestinal mucosa showed a visible difference between DMSO and AG490 larvae, where folds thickness appears diminished, consistently with an inhibition of proliferation (Fig. 3.12 C-C'). These results demonstrate that the intestinal proliferation, aimed at regulating mucosal folds formation during development, depends on Stat3 .

3.4 Generation of a *stat3* KO zebrafish mutant

Recent work has shown that bacterial type II CRISPR systems can be adapted to create guide RNAs (gRNAs) capable of directing site-specific DNA cleavage by the Cas9 nuclease in vitro. This system can function *in vivo* to induce targeted genetic modifications in zebrafish embryos with efficiencies comparable to those obtained using ZFNs and TALENs for the same genes (Hwang et al., 2013). With the aim of confirming our data and deeply investigate Stat3 role during zebrafish development, we decided to generate a Stat3 KO mutant, specifically depleting those functional domains that are known to be indispensable for its function: the DNA binding and the C-terminal transactivation domains. The sgRNA was generated to target exon 14, which is encoding for the central portion of the DNA binding domain.

3.4.1 stat3_ex14 sgRNA injection transiently mimics homozygous condition

The sgRNA specifically targeting *stat3* gene was generated using the online Chop Chop tool, that permits the design of single guides with the minimal probability of off-targets.
A mix containing 68 ng/uL of *stat3 _ex14* sgRNA together with 200 ng/uL Cas9 protein was injected into 1-2 cell stage embryos to induce the mutagenesis.
The efficiency of the *stat3_ex14* sgRNA was tested by genotyping a portion of the injected F0 embryos, through PCR and Heteroduplex Motility Assay (HMA). Surprisingly 80% of the injected embryos showed complete elimination of the WT allele band, suggesting an incredibly high mutagenesis rate (Fig. 3.13 A). This was also confirmed by PCR sequencing electropherogram, that starting from the PAM region of the targeted gene portion, presents multiple overlapping peaks with equivalent intensity (Fig. 3.13 B).
Very interestingly, this transient *stat3* KO, showed a specific redundant phenotype in the F0 injected larvae, starting from 3 dpf that rapidly led to death at 5 dpf. 4 dpf F0 injected larvae show defects in those tissues where Stat3 activity is present during the first day post fertilization in the reporter fish; they have smaller head, smaller eye and thinner gut, generally showing a delay in development (Fig. 3.13 C-C'). Moreover, injection of the *stat3_ex14* sgRNA into 1 cell stage *Tg(7xStat3-Hsv.Ul23:EGFP)*reporter embryos, caused a significant decrease of the EGFP production in the telencephalon already at 22 hours post injection (hpi) (Fig. 3.13 D-D').
Based on our previous findings Stat3 is maternally inherited by the oocyte, since *stat3* signaling pathway activation is detectable diffusely before the midblastula transition (MBT), the specific developmental stage at which the embryo activates the zygotic transcriptional machinery. Recently, Liu et al. publication revealed that Cas9 containing NLS signal has the ability to interact with mRNA in nucleus and induces suppression of mRNA translation via the roadblock mechanism in cytoplasm, suggesting a dual role for Crispr Cas9 technique in targeting both DNA and mRNA. According to those informations, the Crispr Stat3 KO phenotype that is detectable in the F0 injected generation, might be due to both depletion of zygotic Stat3, and maternally inherited Stat3 mRNA.

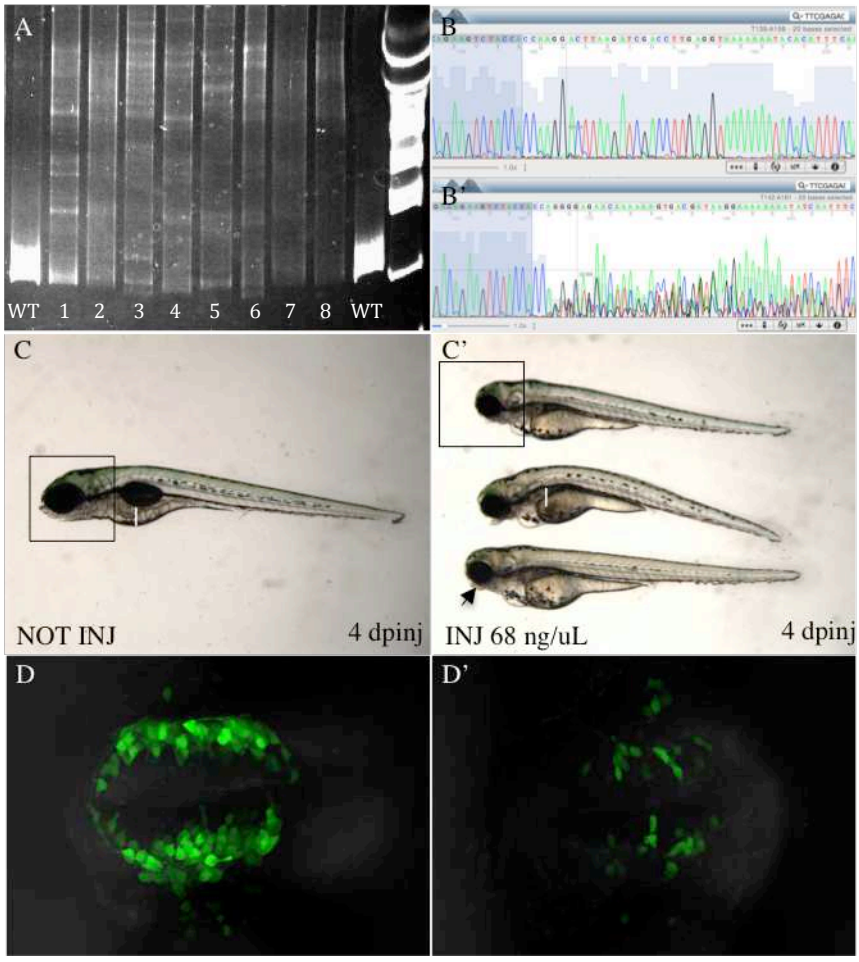

Figure 3.13 ***stat3_ex14* sgRNA injection transiently mimics Stat3 KO.** HMA on F0 embryos injected with *stat3_ex14* sgRNA and Cas9 protein (A) demonstrates complete abolishment of WT allele band in 80% of the samples (A). Sanger sequencing shows double overlapping peaks in the genomic region targeted by *stat3_ex14* sgRNA in the injected embryo (B') while single distinguishable peaks are present in the WT control sequence (B). 4 dpf Crispr transient KOs (C') show redundant developmental defects when compared to the un-injected control (C): smaller head (square), thinner gut (white bar), smaller eye (arrow). *Tg(7xStat3-Hsv.Ul23:EGFP)*injected with *stat3_ex14* sgRNA and Cas9 protein (D') reveal a strong inhibition of the endogenous Stat3 pathway signal (D) in the 24 hpf telencephalon.

3.4.2 Isolation of a stat3Δ8bp mutant fish

With the aim to isolate a Stat3 stable mutant, F0 embryos injected with *stat3_ex14* sgRNA were raised to adulthood and outcrossed with WT fish to screen the F1 progeny for useful mutations. First screen in the F1 generation was performed with HMA as described in the Materials and Methods section, and later on, F1 progeny from the identified F0 founders was genotyped through PCR and Sanger sequencing to look for *non sense* mutations. Among them, F0 #3 founder fish showed by HMA a unique heteroduplex migration pattern, that after sequencing was identified as a 13 bp deletion together with a 5 bp insertion probably due to a DNA polymerase partial repair activity.

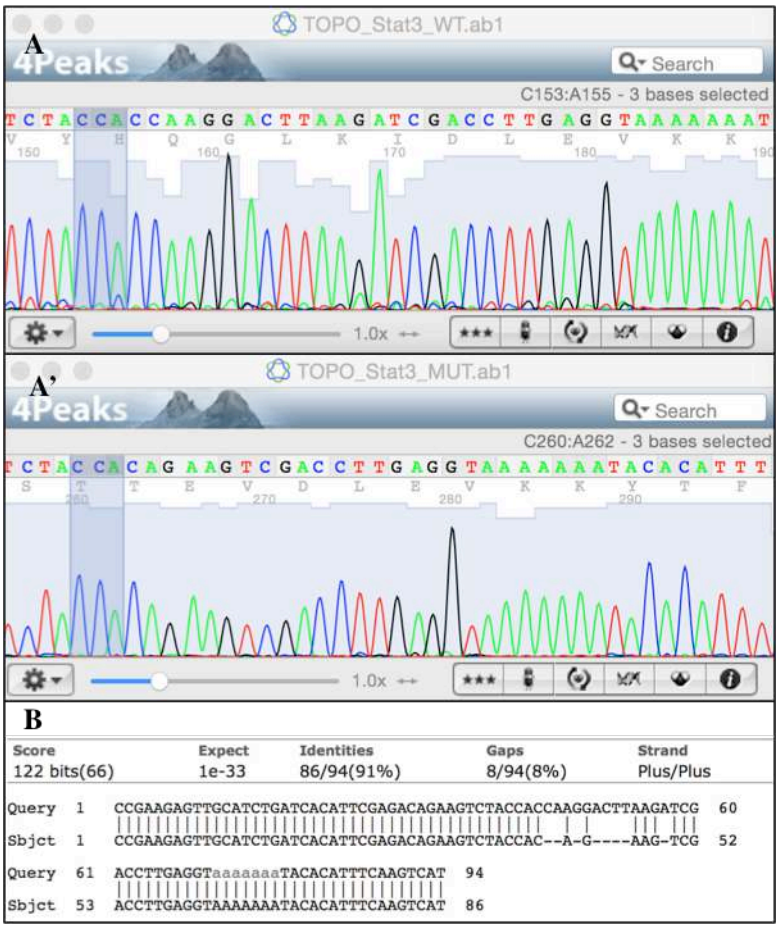

Figure 3.14 Sanger sequencing of pTOPO-*stat3WT* and pTOPO-*stat3Δ8bp*. Electropherogram of respectively pTOPO-*stat3WT* and pTOPO-*stat3Δ8bp* plasmids (A;A'). BLAST output for pTOPO-*stat3WT* and pTOPO-*stat3Δ8bp* two sequences alignment showing the 8 bp gap.

Together these genomic modifications lead to an 8 bp deletion inside Stat3 exon 14 sequence. PCR from heterozygous mutants was then cloned into a pCR™2.1-TOPO®TA plasmid following manufacturer's instructions and sequenced confirming that a total of 8 bp had been deleted from *stat3* exon14 in F0 #3 founder progeny (Fig. 3.14).

The 8 bp genomic deletion causes the formation of a Stop codon at aa 456 depleting both DNA Binding and Transactivation domains (Fig 3.15), thus resulting in a truncated Stat3 protein that lacks 354 of the 810 amminoacids. The isolated mutant was then used for further investigations.

WT Stat3 810 AA

MAQWNQLQQLETRYLEQLYHLYSDSFPMELRQFLAPWIESQDWAYAAN
KESHATLVFHNLLGEIDQQYSRFLQENNVLYQHNLRRIKQHLQSKYLEK
PMEIARIVARCLWEEQRLLQTATTAQQDGQVAHPTGTVVTEKQQILEHN
LQDIRKRVQDMEQKMKMLENLQDDFDFNYKTLKSAGELSQDLNGNSQA
AATRQKMSQLEQMLSALDQLRRQIVTEMAGLLSAMDFVQKNLTDEELA
DWKRRQQIACIGGPPNICLDRLETWITSLAESQLQIRQQIRKLEELQQKV
SYKGDPIIQHRPALEEKIVDLFRNLMKSAFVVERQPCMPMHPDRPLVIK
TGVQFTTKVRLLVKFPELNYQLKIKVCIDKESGDVAAIRGSRKFNILGTN
TKVMNMEESNNGSLSAEFKHLTLREQRCGNGGRTNSDASLIVTEELHLI
TFETEVYHQGLKIDLETHSLPVVVISNICQMPNAWASILWYNMLTNHPK
NVNFFTKPPVGTWDQVAEVLSWQFSSTTKRGLTIEQLTTLAEKLLGPCV
NYSGCQITWAKFCKENMAGKGFSFWVWLDNIIDLVKKYILALWNEGYI
MGFISKERERAILSPKPPGTFLLRFSESSKEGGITFTWVEKDINGKTQIQS
VEPYTKQQLNSMSFAEIIMGYKIMDATNILVSPLVYLYPDIPKEEAFGKY
CRPEAHPDTEFPDTGCVTQPYLKTKFICVTPCPSVFMDFPDSELLGNGFP
GTNSGNTSDLFPMSPRTLDSLMHNEAAEANPGPLESLTLDMELSSDHAS
PMREGFAASTVSDMDTCRNA Stop

Stat3Δ8bp 456 AA

MAQWNQLQQLETRYLEQLYHLYSDSFPMELRQFLAPWIESQDWAYAAN
KESHATLVFHNLLGEIDQQYSRFLQENNVLYQHNLRRIKQHLQSKYLEK
PMEIARIVARCLWEEQRLLQTATTAQQDGQVAHPTGTVVTEKQQILEHN
LQDIRKRVQDMEQKMKMLENLQDDFDFNYKTLKSAGELSQDLNGNSQA
AATRQKMSQLEQMLSALDQLRRQIVTEMAGLLSAMDFVQKNLTDEELA
DWKRRQQIACIGGPPNICLDRLETWITSLAESQLQIRQQIRKLEELQQKV
SYKGDPIIQHRPALEEKIVDLFRNLMKSAFVVERQPCMPMHPDRPLVIK
TGVQFTTKVRLLVKFPELNYQLKIKVCIDKESGDVAAIRGSRKFNILGTN
TKVMNMEESNNGSLSAEFKHLTLREQRCGNGGRTNSDASLIVTEELHLI
TFETEVYHRSRP Stop

Figura 3.15 Amminoacidic sequence of Stat3-WT and Stat3Δ8bp proteins.

3.4.2.1 Stat3 depletion causes intestinal and neural proliferation failure in the developing larvae

According to previous findings, Stat3 transcription factor regulates proliferation both in the developing nervous system and gut tissues. The Stat3Δ8bp mutation abolishes Stat3 zygotic gene expression in homozygous fish, but the embryo still translates Stat3 protein starting from maternal mRNAs of the WT allele.

Moreover, in contrast with the transient activation of the signaling pathway in response to time specific phosphorylation inputs by the Jak2 kinase, STAT3 protein presents an incredibly low turnover rate, that in COS-6 fibroblasts cell culture, reaches 24 hours of half-life (Siewert et al., 1999).

Consistently with that, the *stat3Δ8bp -/-* mutant does not show an early phenotype, but starts to reveal behavioural and morphological changes only at 4 dpf. At this stage KO fish are recognizable because of a bigger swim bladder and reduced motility reaction upon exogenous mechanical stimulation, possibly due to neural damages (Fig. 3.16 A-A'').

Based on our previous findings, STAT3 acts as a permissive factor of cell division during development, in particular in the intestine where Stat3 inhibition causes loss of proliferation and mucosal folds. To assess whether *stat3* genetic KO causes a similar phenotype, we performed a P-H3 proliferation assay on the 5 dpf heterogeneous progeny of two *stat3Δ8bp +/-* fish. Strikingly, ¼ of the samples, that was later on identified as homozygous through genotyping, showed a significantly visible reduction of the proliferation both in the intestinal duct, and in the central nervous system (Fig. 3.16 B-B').

According to this, we definitely correlated Stat3 activity with the mitotic rate responsible for primordial intestine and nervous system development in zebrafish larvae.

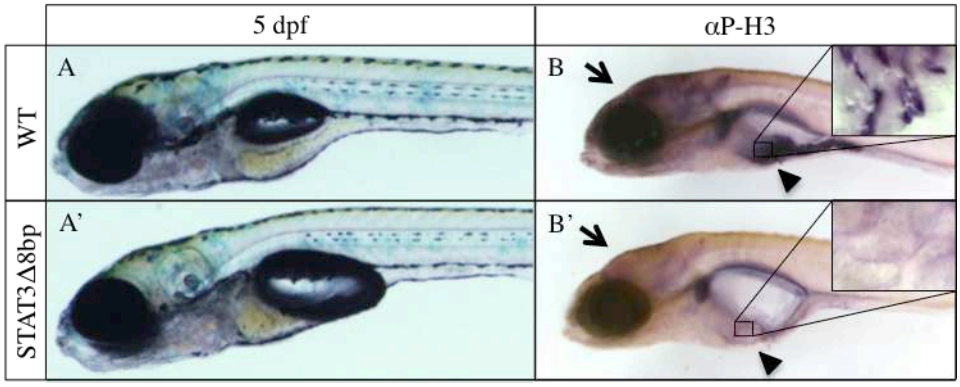

Figure 3.16 Stat3 KO presents loss of intestinal and neural proliferation at 5 dpf. *stat3Δ8bp -/-* larvae at 5 dpf (A') are recognizable by the overgrown swim bladder from the WT and *stat3Δ8bp +/-* (A). α P-H3 proliferation assay clearly shows the inhibition of cell division in the intestine (arrowhead) and brain (arrow) of the *stat3Δ8bp -/-*developing larvae (B') with respect to the WT (B).

3.4.2.2 stat3 KO mutant lacks intestinal folds

A more detailed analysis on *stat3Δ8bp -/-* mutant intestine, performed by sectioning paraffine embedded 6 dpf larvae and staining them with hematoxylin and eosin, revealed a significant reduction in the height of the intestinal folds (Fig. 3.17). This reduction is possibly due to the previously reported abolishment of intestinal proliferation, which highly affects the correct development of the intestinal mucosa.

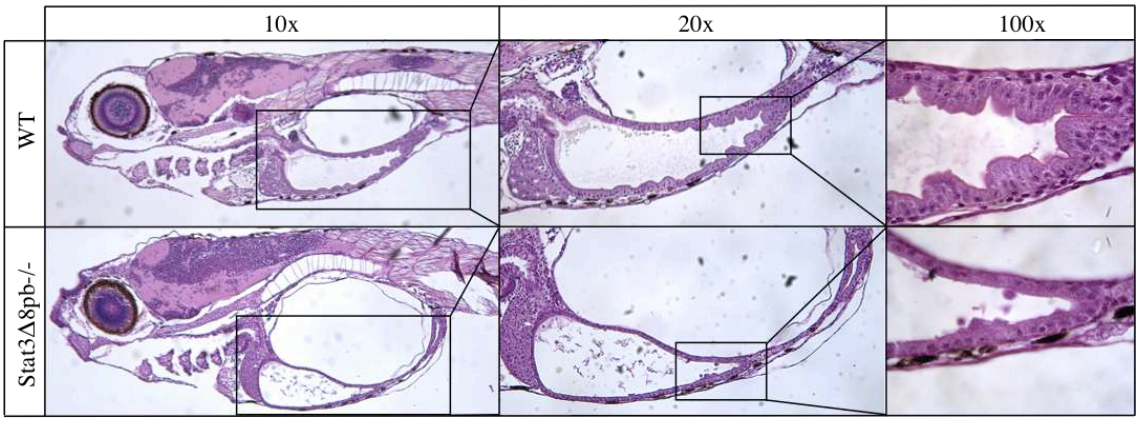

Figure 3.17 Stat3 KO zebrafish lack intestinal folds. H/E staining on 6 dpf paraffin embedded larvae. While the WT larva shows normal intestinal mucosa with columnar cells and developing folds, the *stat3Δ8bp -/-* mutant fish shows a single layered cubical undifferentiated epithelium, which reveals a complete abolishment of intestinal folds formation.

Moreover, the normal columnar intestinal epithelium, which is visible in the WT larvae, is replaced by a single-layer of cubical undifferentiated cells. This result is consistent with the previously reported abolishment of intestinal mucosa development in zebrafish larvae treated with AG490 Stat3 pathway inhibitor.

3.4.2.3 stat3Δ8bp -/- mutation is lethal at late larval stages

To investigate whether Stat3 KO is associated with a lethal phenotype at late larval stages, we performed a life span assay, by monitoring the progeny obtained by in-crossing two *stat3Δ8bp +/-* fish. Notably, starting from a pool of 100 embryos, 22 larvae died at 8 dpf. Subsequent genotyping, performed following our optimized genotyping strategy (see Materials and Methods, section 2.15) confirmed that no *stat3Δ8bp -/-* fish survive until juvenile stage.
Most probably the lack of intestinal folds leads zebrafish homozygous larvae to death shortly after yolk sack consumption.

3.5 Stat3 regulates proliferation through mtDNA transcription

3.5.1 Mitochondrial Stat3 acts as a transcription factor for mtDNA

STAT3 protein was discovered together with other members of the Stat family of proteins in 1997 (Ihle et al.,1997) and its nuclear transcriptional activity has been investigated for the following 12 years, until 2009 when the first evidence of a mitochondrial form of Stat3 was observed by Wegrzyn et al. Recently, in 2016, mitoStat3 has been rediscovered as a mediator of proliferation for embryonic stem cells culture, through its regulation of mitochondrial genes transcription (Carbognin et al., 2016).

To verify whether the Stat3 activity on cell division could be due to its mitochondrial functions, we injected a mitochondrial targeted form of murine Stat3 mRNA, that also included a Nuclear Export Sequence (MLS_mStat3_NES), into 1 cell stage embryos. This specific form of Stat3, was previously shown to completely lack the nuclear transcriptional activity (Carbognin et al., 2016). Interestingly, injected embryos showed a significant enrichment at 48 hpf in mitochondrial gene expression assessed by ISH using mt_nd2 probe, which is here used as a hallmark of mitochondrial transcription because of the polycistronic nature of mtDNA (Fig. 3.18 A-A'). Moreover, the α P-H3 proliferation assay confirmed a notable increase in the mitotic rate of those same tissues where mitochondrial transcription is enhanced: the optic tectum and the haematopoietic tissue (Fig. 3.18 C-C').

Coherently, AG490 inhibitor, that prevents Jak2 phosphorylation to Stat3 Y705 and thus Stat3 dimers stabilization, reduced *mt-nd2* expression and cell division, with respect to the untreated 72 hpf control (Fig. 3.18 B-B'; D-D').

These data indicate that mitoStat3 and the phosphorylation of its Tyr 705 are needed for mitochondrial DNA transcription aimed at regulating proliferation in the brain and in the haematopoietic tissue of developing embryos. Interestingly, mitochondrial gene expression localizes in those tissue where also the Stat3 nuclear activity was reported by the *Tg(7xStat3-Hsv.Ul23:EGFP)* transgenic embryo, suggesting a dual role in those cells for the transcription factor subsequent to Jak2 Y705 phosphorylation.

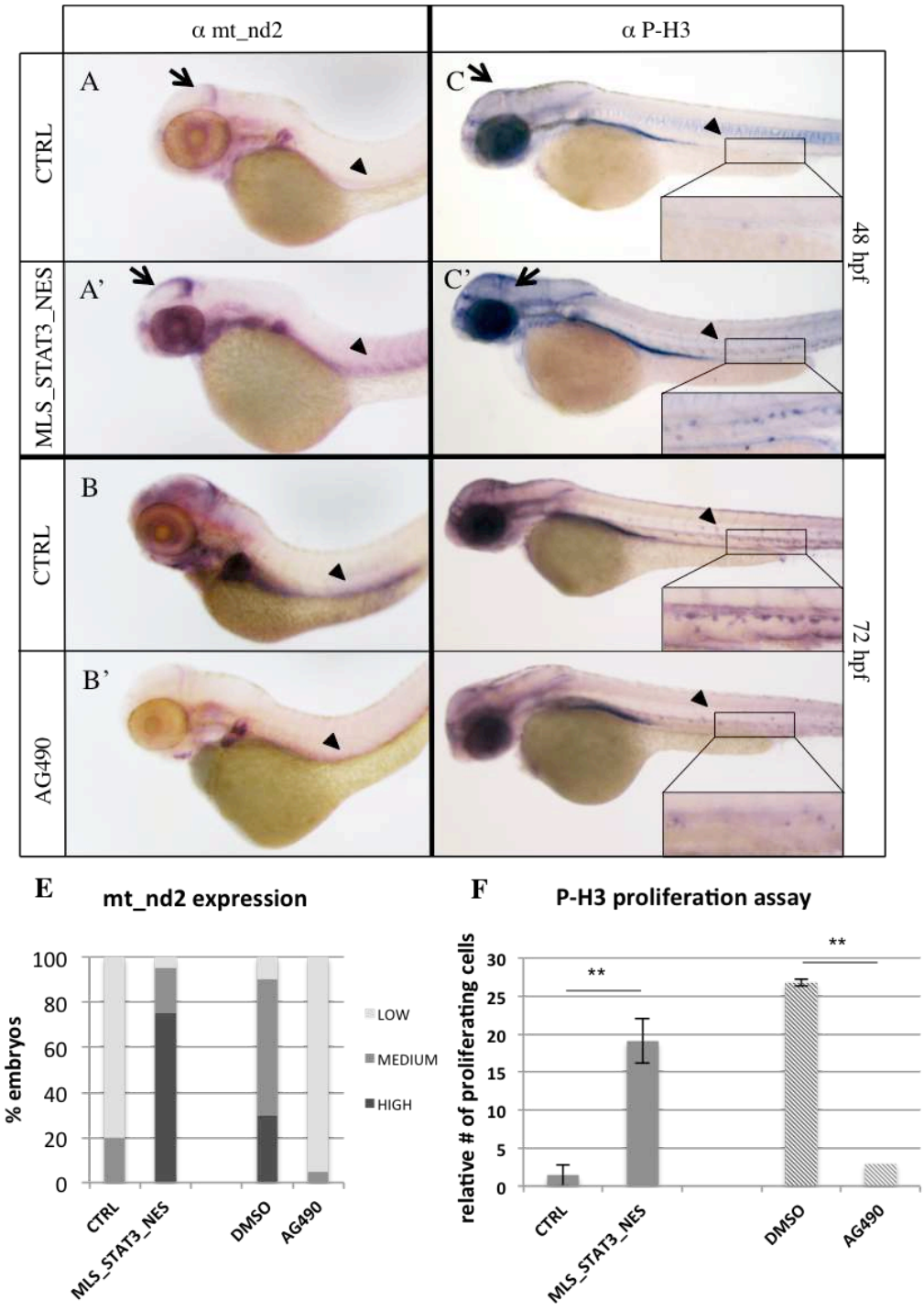

Figure 3.18 A mitochondrial form of Stat3 promotes cell division through mtDNA transcription. 48 hpf embryos injected with MLS_STAT3_NES display increased *mt_nd2* mRNA expression (A') and P-H3 mitosis marker (B') in the brain (arrow) and in the haematopoietic tissue (arrowheads), when compared to un-injected controls (A,C). Endogenous mt_nd2 expression (B) is significantly reduced when embryos are treated with AG490 Stat3 inhibitor (B'); the same treated embryos show a clear inhibition of proliferation (D') if compared to DMSO treated controls (D). Graphical representation of the percentages of embryos displaying high, medium or low expression of *mt_nd2* (E). Graphical representation of the amount of proliferating cells scored in the haematopoietic tissue (F).

3.5.2 Two post-translational modifications are necessary to mitoStat3 transcriptional function

As mentioned before, to date, two phosphorylation sites are known to be important for STAT3 activity (Y705 and S727), and in particular S727 had been previously related to mitochondrial complex I and II efficiency (Wegrzin et al., 2009). Our previous findings suggest Stat3 driven mitochondrial genes expression to be directly correlated to proliferation, however still nothing is known about the post-translational modifications that are required for mitoStat3 transcriptional activity. With the aim of investigating which of the two phosphorylation sites is necessary for STAT3 mitochondrial genes expression, we generated two mutated forms of murine STAT3 mRNA (Y705F and S727A) that do not allow phosphorylation, and injected them into 1-2 cell stage embryos. ISH with mt_nd2 probe revealed mitochondrial genes expression to be almost completely abolished in the optic tectum, by injection of the two STAT3 mutant mRNAs with respect to the control injected with STAT3 WT mRNA (Fig. 3.19). These data prove that both STAT3 Y705 and S727 phosphorylation sites must be available to allow mitoSTAT3 transcriptional function .

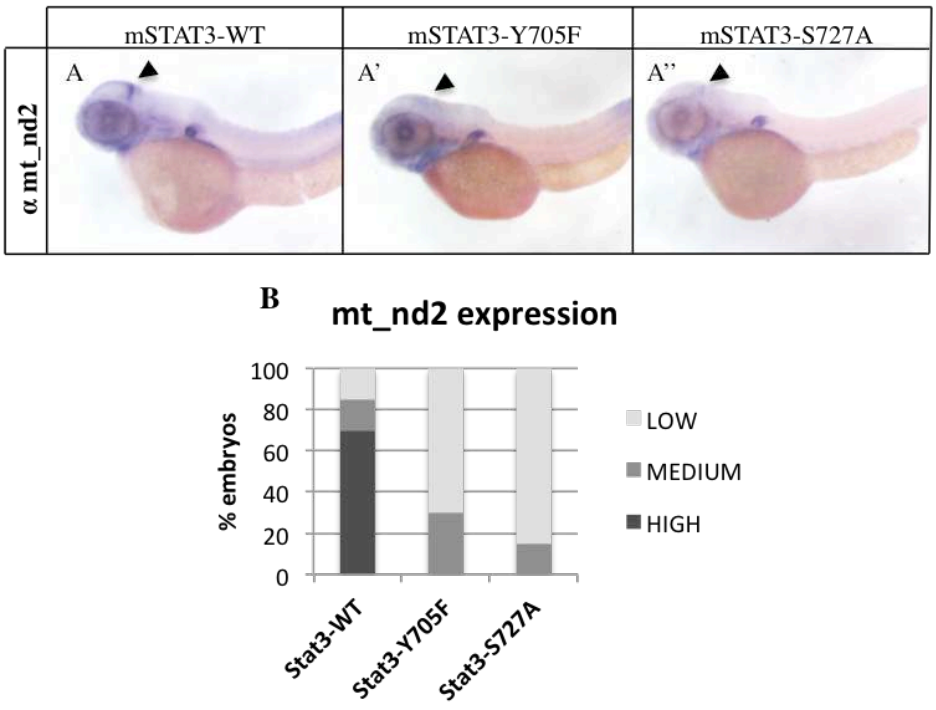

Figura 3.19 Y705 and S727 phosphorylation sites are necessary for Stat3-driven mtDNA transcription. Injection of murine Stat3-WT and mutated Stat3 Y705F and S727A mRNAs into 1 cell stage embryos. At 48 hpf *mt_nd2* expression results significantly higher in the optic tectum (arrowhead) of embryos injected with Stat3-WT mRNA (A) when compared to the Stat3-Y705F (A') and Stat3-S727A (A'') injected embryos. Graphic representation of the percentage of embryos showing high, medium and low *mt_nd2* expression after injection (B).

3.5.3 mitoStat3 regulates proliferation through mtDNA transcription

Stat3-dipendent gene regulation has been extensively studied in many different biological context, however, in some previous publications mitoStat3 was found to be associated with the inner mitochondrial membrane (Tammineni et al., 2013) and to regulate the activity of the complexes of the electron transport chain (ETC) by direct interaction with them, thus influencing mitochondrial respiration (Yang and Ricorn, 2016) . Specifically, *STAT3-/-* cells significantly reduce ETC activity of complex I and II, while MLS_STAT3 completely restores their functionality (Wegrzyn et al., 2009). These findings posed the question whether some STAT3 functions might be independent of its activity as transcription factor.

Even thought our previous findings demonstrate a link between mitoStat3 transcriptional activity and proliferation, we cannot exclude an interaction of Stat3 with the ETC complexes and, indirectly, with mitosis.

To test additional contribution in the promotion of cell division other than mitoStat3-dependent transcriptional activation, we decided to repeat our previous experiment of MLS_STAT3_NES injection while blocking mtRNA Polymerase activity, thus completely avoiding any mitochondrial gene expression.

To do that we administrated a known mtRNA Polymerase inhibitor (Balapiravir) (Feng et al., 2016) starting from 8 hpf to MLS_STAT3_NES injected embryos, in order to compare proliferation rate with respect to the MLS_STAT3_NES untreated controls at 48 hpi. αP-H3 IHC revealed that injected embryos, when treated with 50 uM Balapiravir, are significantly less proliferating when compared to the injected untreated control, confirming that mitoSTAT3 regulates proliferation exclusively through mtDNA transcription, thus independently from any potential interaction with the ETC complexes (Fig. 3.20 C-C''). As a control experiment, we performed ISH using the *mt_nd2* probe, that represents mtDNA transcription. Balapiravir was observed to efficiently block

mitochondrial genes expression in the MLS_STAT3_NES injected embryos, when compared to the MLS_STAT3_NES injected, untreated fish (Fig. 3.20 D-D'').

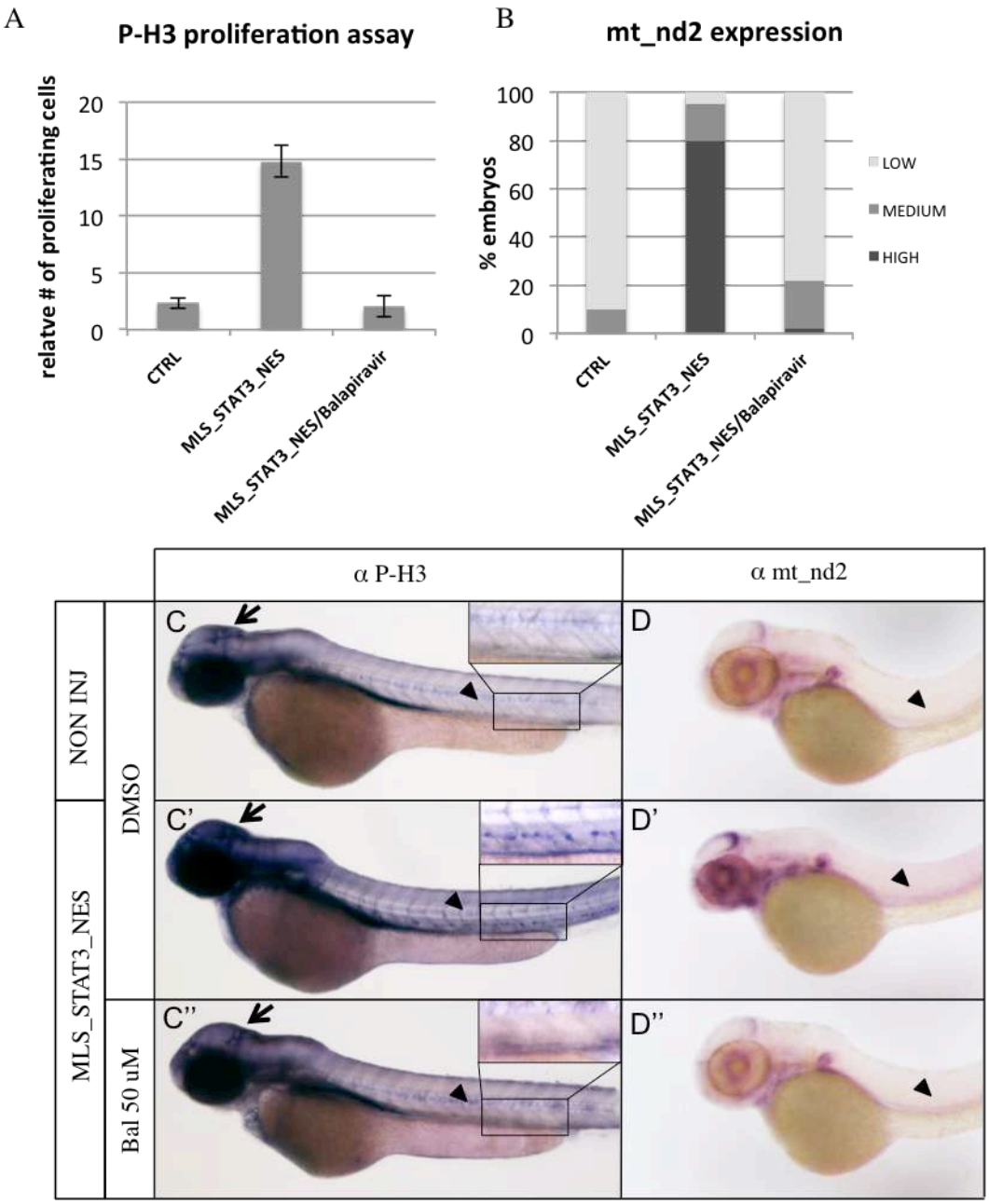

Figure 3.20 mitoStat3 promotes proliferation exclusively through its transcriptional activity. Graphic representation on the percentages of embryos displaying high, medium and low expression of *mt_nd2* (B) and the number of proliferating cells scored in the haematopoietic tissue (A) upon indicated injection and treatment conditions. MLS_STAT3_NES injected embryos show a promotion of both proliferation (C') and *mt_nd2* gene transcription (D') with respect to the un-injected controls (B,C). However, Balapiravir administration from 8 to 48 hpf to MLS_STAT3_NES injected embryos almost completely abolishes both mtDNA transcription (D'') and cell division (C'').

3.6 *Tg(7xStat3-Hsv.Ul23:EGFP)* marks Cancer Stem Cells in *apc*-zebrafish model for colorectal cancer

The Janus kinases (JAKs) and signal transducer and activator of transcription (STAT) proteins, particularly STAT3, are among the most promising new targets for cancer therapy. In contrast to the transient nature of STAT3 activation in normal cells, many human solid and haematological tumours harbour constitutive STAT3 activity. Some examples are leukemias, lymphomas, multiple myeloma, brain, breast, colon and pancreatic tumours. Importantly, evidences reported in a variety of tumor cell lines, as well as primary human tumours, strongly implicates aberrantly active STAT3 function in tumor formation, invasion and metastasis (Yue and Turkson, 2009). Novel approaches to investigate JAK–STAT3 in tumours represent important tools for development of therapeutic strategies in the treatment of cancer.

With the aim to prove the great potential of the novel *Tg(7xStat3-Hsv.Ul23:EGFP)* transgenic fish in STAT3-targeted cancer research, we crossed the reporter line with the known *apc* zebrafish genetic model for colorectal cancer, in order to observe the dynamical activation of Stat3 signaling pathway in a hyperplastic tissue.

Loss of human APC function has been in fact defined as the initial step toward colon cancer formation: inactivating mutations of *APC* were identified in familial adenomatous polyposis (Groden et al., 1991); notably, even in sporadic cases of colorectal carcinomas, ~85% of the tumours carried APC-truncating mutations.

Through its role in targeting β-catenin for ubiquitination and degradation, loss of APC leads to constitutive activation of the Wnt pathway with accumulation of β-catenin in the nucleus and activation of downstream Wnt target genes transcription.

Mice homozygous for *Apc* mutations are embryonic lethal, whereas heterozygous mice develop tumours in the small intestine (Ward and Devon-Henneman, 2004). Similarly, fish carrying an *apc* mutant allele display similar phenotypes: homozygous animals die during development, while heterozygous animals develop spontaneous tumours later in life, primarily in the liver and intestine (Haramis et al., 2006). The tight conservation of APC as a crucial tumor suppressor further supports the validity of using zebrafish to model digestive tract cancer.

Heterozygous *apc* mutants are known to develop colorectal cancer starting from 12 months of age. The *Tg(7xStat3-Hsv.Ul23:EGFP)* harbouring *apc+/-* mutation were

sacrificed at 14 mpf, and paraffin embedded intestinal sections were stained with Hematoxylin-Eosin to histologically compare normal and hyperplasic tissue. Normal adult intestine displays ordered periodic arrangement of the crypts/villi structures, that is, in contrast, completely lost in the *apc*- tissue, where large outgrowths resembling mammalian adenomatous polyps accumulate together with ramification of the villi (Fig. 3.21 A-A'). Importantly, with our experimental results we could confirm the previous observations that the morphology and histopathological features of the neoplasia observed in fish resemble those found in humans (Haramis et al., 2006) (Amatruda et al.,2002).

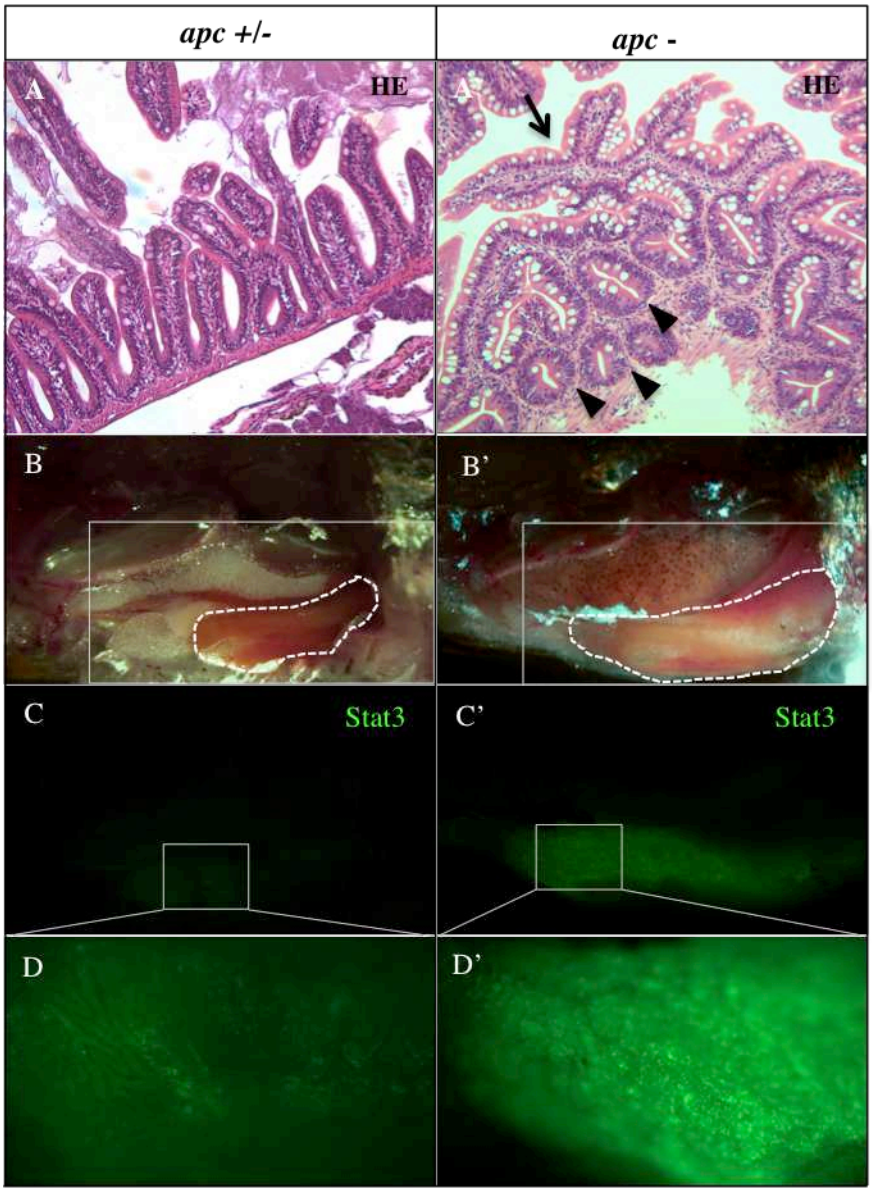

Figure 3.21 Stat3 pathway is ectopically active in *apc*- hyperplasic zebrafish intestine. Histology of an *apc+/-* normal adult intestine and an *apc*- adenocarcinoma stained with HE. Normal adult intestine displays ordered periodic arrangement of the crypts/villi structures (A). The tumour (A') shows typical gland-like polyps that resemble the human adenomatous polyps (arrowheads), and overgrown ramifications of villi (arrow). Adult zebrafish organs as they appear after epidermis and muscle removal: the area delimited by the striped line indicates the intestine dimension: the normal intestine (B) is much smaller than the hyperplasic one (B'). *in vivo* endogenous EGFP detected by fluorescence microscopy in Tg(7xStat3-Hsv.Ul23:EGFP)/apc+/- fish: normal intestine (C,D) expresses a notably reduced amount of Stat3 reporter signal with respect to the tumour (C',D'), thus indicating Stat3 pathway constitutive activation in *apc*-driven colorectal cancer.

During sacrifice procedure, the euthanized fish were observed through fluorescence stereomicroscopy, to have a preliminary *in vivo* insight on the relative intestinal EGFP production displayed by the normal and the hyperplasic organ at 14 months of age. Notably, those *Tg(7xStat3-Hsv.Ul23:EGFP)/apc+/-* fish that presented ventral detectable buds, once sectioned, revealed overgrown intestine and higher accumulation of endogenous EGFP with respect to normal-appearing ones (Fig. 3.21 B-D').

Together with its roles in regulating cytokine-dependent inflammation and immunity, Stat3 protein is central in determining whether immune responses in the tumour microenvironment promote or inhibit cancer (Yu et al., 2009). Persistently activated Stat3 in fact, not only increase tumour cell proliferation, survival and invasion while suppressing anti-tumour immunity, but also mediates tumour-promoting inflammation. STAT3 has therefore a dual role in tumour inflammation and immunity by promoting pro-oncogenic inflammatory pathways, including nuclear factor-κB (NF-κB) and interleukin-6 (IL-6)–GP130–Janus kinase (JAK) pathways, and by opposing STAT1- and NF-κB-mediated T helper 1 anti-tumour immune responses (Yu et al., 2009).

Because of this dual role, there was the need for us to correlate a specific role in the tumor microenvironment, to the observed Stat3 constitutive activation in our *Tg(7xStat3-Hsv.Ul23:EGFP)/apc+/-* model: is Stat3 activation reported by the novel *Tg(7xStat3-Hsv.Ul23:EGFP)*line due to its tumor-promoting pro-inflammatory action or is it related to Stat3-driven proliferation increment?

To exclude Stat3 role as inflammation promoter we decided to first check the inflammation state in the *apc*- tumour at different timings through an Alcian Blue staining on *apc*- 6 mpf, 12 mpf and 18 mpf intestinal sections. Alcian blue stains acidic

polysaccharides that compose the mucine, the main product of the intestinal secreting Goblet Cells, that is normally secreted by those gastrointestinal epithelial cells in response to bacterial infection, as a defence layer. Increased intestinal levels of mucine are thus correlated to diffused inflammation (Kim and Ho, 2010). Conversely to many other intestinal pathologies, the *apc*-driven carcinoma usually eliminates goblet cells, and therefore mucine production, during adenomatous polyps progression, thus excluding the predominance of the inflammation process as a driver of tumour progression (Amatruda et al., 2002). Unexpectedly, the alcian blue staining displayed a time-dependent increase of the extracellular mucine accumulation, thus revealing a strong inflammation condition in the hyperplasic intestine (Fig. 3.22).

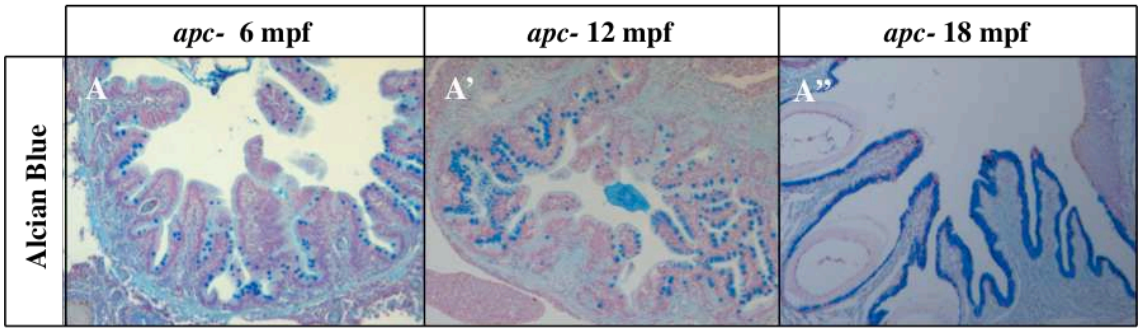

Figure 3.22 **Mucine accumulates during tumour growth in *apc*- driven adenocarcinoma.** Alcian Blue staining on sections of hyperplasic intestine at 6, 12 and 16 mpf. Blue pigment stains Goblet Cells and extracellular mucine as marker of inflammation in zebrafish intestine revealing that inflammation is proportional to tumour progression in the apc-driven adenocarcinoma.

Our second experimental approach tended to directly correlate Stat3 over-expression in the tumour with the increment in proliferation that is normally detectable in *apc*-driven intestinal hyperplasia.

IF staining using anti-PCNA indicated ectopic proliferation in the portion of the intestinal tissue that displayed the classical *apc*- histology, while in the normal tissue proliferation resides only at the base of the inter-villus pocket. Notably, Stat3 reporter signal, stained with anti-EGFP Ab, accumulates in the same portion of the subsequent section, while in the regular crypt/villus structure Stat3 is only active in few cells at the base of the inter-villus pocket (Fig. 3.23 A,A''). Strikingly, double IF revealed co-localization between the EGFP positive cells and the mitosis marker, indicating that all Stat3 expressing cells also

are proliferating in the tumour microenvironment; this finally excludes the possibility of Stat3 signal to be related to the inflammation process in *apc*-driven tumorigenesis.

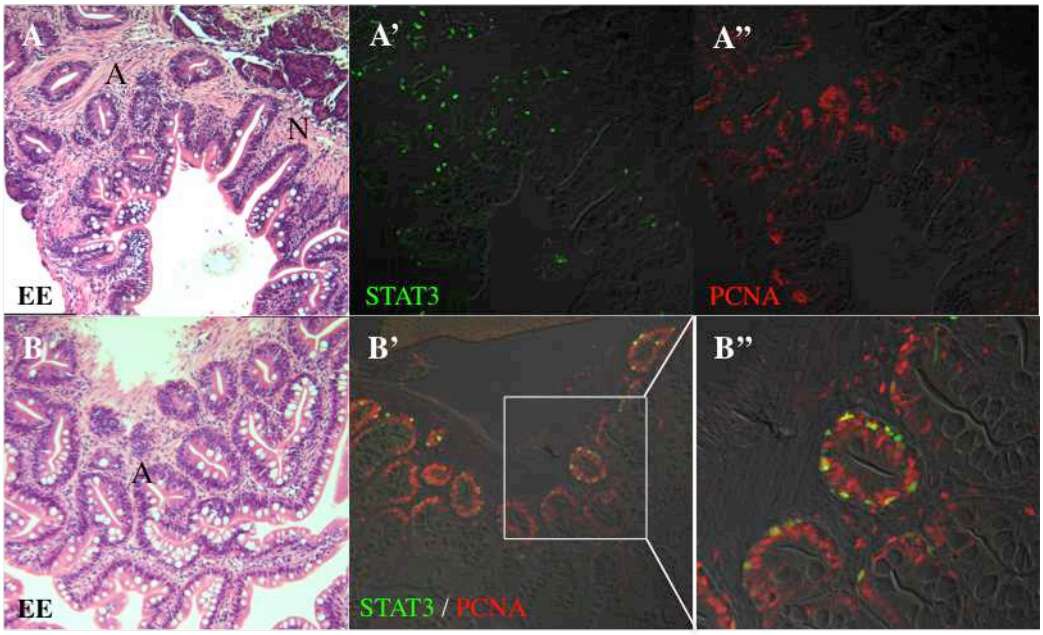

Figure 3.23 Stat3 pathway marks proliferating CSC in the *apc*-driven intstinal tumour. (A) HE staining on an adult zebrafish intestinal section harbouring both hyperplasic adenomas (indicated by letter A) and normal tissue (letter N). IF staining on an adjacent intestinal section with anti-EGFP Ab shows Stat3 reporter signal accumulation in the adenomatous polyps while in the normal tissue Stat3 marks only few cells at the base of the inter-villus pocket (A'). anti-PCNA proliferation marker shows that the adenomatous tissue is much highly mitotic with respect to the normal tissue in the same section (A''). (B) HE staining on an adult *apc*-intestinal tumour, where the tissue is almost completely adenomatous: double IF with anti-EGFP and anti-PCNA show that in the polyps all Stat3 expressing cells are proliferating, and surrounded by other mitotic but not Stat3 positive cells (B',B'').

Again Stat3 signaling pathway is only active in a restricted portion of the proliferating cells inside the adenomatous polyps (Fig. 3.23 B''), indicating that the transcription factor is not simply a mitosis marker.

Previous experiments have identified Stat3 positive cells as Crypt Base Columnar cells, an adult stem cell that actively proliferate giving birth to the TA population, in physiological conditions in the zebrafish intestine. In the tumour microenvironment, the so called Cancer Stem Cells represent the CBC counterpart in pathological conditions. Differently from other Cancer Cells, they possess characteristics associated with normal Stem Cells,

such as the ability to give rise to all cell types found in a particular cancer sample, therefore being tumorigenic.

The origin of CSCs is an active research area. Origin hypotheses include mutants in developing stem or progenitor cells, mutants in adult stem cells or adult progenitor cells and mutant, differentiated cells that acquire stem-like attributes. These theories often focus on a tumour's "cell of origin". In particular, the theory associating adult stem cells (ASC) with tumor formation is most often associated with tissues with a high rate of cell turnover such as the skin or gut. In these tissues, ASCs are candidates because of their frequent cell divisions in conjunction with their long lifespan. This combination creates the ideal set of circumstances for mutations to accumulate, which is the primary factor that drives cancer initiation (López-Lázaro, 2015). Therefore, development of specific therapies targeted at CSCs holds hope for improvement of survival and quality of life of cancer patients, especially for patients with metastatic disease.

These insights together reveal that Stat3 signaling pathway is likely a marker of stemness, generally being active in CBCs and CSCs in zebrafish cancer models. Therefore, the novel *Tg(7xStat3-Hsv.Ul23:EGFP)* reporter line represents a useful tool for the development of new therapeutic approaches towards Stat3-targeted cancer research.

4 CONCLUSIONS and DISCUSSION

4.1 The novel Stat3 zebrafish reporter

STAT3 is the most ubiquitous of the STATs, being activated by a wide variety of cytokines and growth factors, and fulfilling roles in many physiological processes such as inflammatory signaling, aerobic glycolysis and immune suppression. Moreover, STAT3 was also the first family member shown to be aberrantly activated in a wide range of both solid and liquid tumours where it promotes tumorigenesis by regulating the expression of various target genes, including cell-cycle regulators, angiogenic factors and anti-apoptotic genes. Paradoxically, while in some circumstances STAT3 signaling induces cell death, direct silencing or inhibition of STAT3 diminishes tumour growth and survival both in animal and human studies.

All these insights give a realistic overview of the complex regulation upstream and downstream of STAT3 signaling pathway that, after 20 years from the first identification of the STAT family of proteins, is still stimulating research interest towards many different scientific issues.

In this work we discuss the generation, characterization and validation of a novel Stat3 reporter line, able to unravel the spatial and temporal activation of the signaling pathway *in vivo* in the zebrafish model organism.

The transgenic cassette we used to generate the *Tg(7xStat3-Hsv.Ul23:EGFP)* reporter, containing 7 repeats of the STAT3 responsive element upstream of an EGFP coding sequence, was stably integrated in the genome of a WT fish through transposase driven recombination. Afterwards, the line was validated through pharmacological and genetic approaches that confirmed its responsiveness to STAT3 transcription factor: knockdown and overexpression approaches showed that the CRP-based zebrafish transgenic line effectively reports *in vivo* Stat3 activity, being able to reflect variations in the signaling with a good sensitivity.

It is important to underline that the *Tg(7xStat3-Hsv.Ul23:EGFP)* we developed, is reporting the transcriptional activity of Stat3 and not its simple expression pattern which could be otherwise easily analysed by in situ hybridization. In fact, given the prominent post-translational regulation that governs its dimerization and shuttling to the nucleus, the presence of Stat3 protein in the cell doesn't *per se* mean that Stat3 is transcriptionally

active in the nucleus. During early embryogenesis for instance, Stat3 mRNA, maternally inherited by the oocyte, is ubiquitously present in the blastoderm at blastula stage (Oates et al., 1999), while, by contrast, at this stage p-Stat3 is only present and active in the dorsal portion of the hypoblast, confirming that Stat3 protein presence is not indicative of Stat3 activity.

Moreover, Stat3 protein was found by in vitro biochemical approaches to have an extremely long half-life, which is in contrast with the transient activation of the pathway that we were able to observe in the reporter during development.

This supports our hypothesis that the novel *Tg(7xStat3-Hsv.Ul23:EGFP)* reporter fish, with its fine spatial and temporal representation of the pathway activity, establish a completely new approach to Stat3 signaling investigation, based on *in vivo* live imaging.

4.2 In vivo analysis of *Tg(7xStat3-Hsv.Ul23:EGFP)* reporter and *stat3Δ8bp* KO mutant reveal Stat3 role during zebrafish development

In the mouse model, STAT3 activity is detected during early postimplantation development, suggesting that STAT3 plays a role in early embryogenesis (Duncan et al., 1997). Coherently, STAT3-deficient mice die early in embryogenesis, prior to gastrulation (Takeda et al., 1997). Specifically, by 7.5 days post-coitum STAT3 mRNA is expressed in the extra embryonic visceral endoderm, which is the principal site of nutrient exchange between the maternal and embryonic environments. The timing of the degeneration of STAT3-/- embryos coincides with the onset of STAT3 expression in visceral endoderm in wild-type mice, suggesting that STAT3-/- lethality may be due to a defect in visceral endoderm function, such as nutritional insufficiency (Akira, 2000).

In their 2002 publication, Yamashita et al. described Stat3 as a key regulator of cell movements during gastrulation in the zebrafish model, assessing that Stat3-KD performed with ATG-MO, severely impairs anterior movement of the axial mesendoderm.

Our results suggest a role for Stat3 pathway during zebrafish early developmental stages and even in oogenesis. In *Tg(7xStat3-Hsv.Ul23:EGFP)* reporter embryos, EGFP fluorescence is detectable, ubiquitously after fertilization in embryos derived from transgenic females crossed to wild- type fish. However, no signal is observed at the same

stage in the offspring of male carriers. This is due to maternal effect that indicates Stat3 pathway to be active during oogenesis, thus depositing Stat3 target mRNAs and proteins in the egg. Consistently with that, *crp-2* mRNA expression is diffusely detected in the zebrafish embryo already at 1 cell stage, suggesting that Stat3 target genes are maternally inherited by the oocyte (Thisse et al., 2004).

Later on, from 24hpf, the *Tg(7xStat3-Hsv.Ul23:EGFP)* embryos display a restricted expression of the reporter signal in the telencephalic region, the inner retina, the optic tectum and the developing gut, where it specifically highlights cells undergoing mitosis.

The molecular mechanisms underlying STAT3 involvement in cell cycle progression were demonstrated using an IL-3-dependent pro-B cell line, BAF/B03 (Fukada et al., 1998). Stat3 was shown to regulate proliferation by promoting transcription. Moreover, it has been shown to be necessary for gp130-mediated G1 to S phase of cell-cycle transition, to be involved in the induction of CDK activators cyclins D, A and cdc25A and in down-regulation of CDK inhibitors. Coherently with these findings, *Tg(7xStat3-Hsv.Ul23:EGFP)* embryos activate their reporter signal dynamically in those tissue portions that are known to be much highly proliferating during specific developmental stages. At 24 hpf the telencephalon is involved in cell divisions and movements that will form the anterior intra-encephalic sulcus. At 48 hpf, in the optic tectum, the radial growth of proliferating cells creates a cell migration/differentiation gradient from the peripheral region of the OT to the center of each lobe. Consistently, in *Tg(7xStat3-Hsv.Ul23:EGFP)* embryos a diffused EGFP gradient fading toward the centre of each tectal lobe, is detectable in the optic tectum, while EGFP mRNA expression is strictly present only in the 3-shaped peripheral portion of the OT, where the mitotic niche is known to be.

This suggests that in the 48 hpf developing OT, the *Tg(7xStat3-Hsv.Ul23:EGFP)* reporter signal carries out an in vivo lineage tracing of cells deriving from the proliferating niche.

In the gut, STAT3 role has to date only been correlated to proliferation in adult pathological conditions such as IBD or CRC; specifically, STAT3 is required for transduction of tumor promoting signals from IL-6 and other cytokines and is important to maintain survival and regenerative capacity of IEC (Grivennikov et al., 2010).

In our *in vivo* model, Stat3 pathway was found correlated to proliferation during intestine development: indeed Stat3 depletion in the *stat3Δ8bp-/-* mutant led to a significant decrease in the cell division rate, that brought to failure of the intestinal folds formation.

Notably, in the *stat3Δ8bp-/-* mutants we were unable to observe phenotypes before 4 dpf, supporting the idea that maternally supplied Stat3 might play a crucial role during early stages of development.

Our results suggest a direct regulation of cell proliferation by Stat3 transcription factor during zebrafish development, a function that seem to be exerted mainly in the nervous system and in the intestine.

4.3 Stat3 is a stemness marker in the adult zebrafish intestine

In adult tissues Stat3 role has been extensively investigated in mice through tissue-specific targeting of STAT3 (Akira, 2000). STAT3 is known to function in vivo in macrophages and neutrophils to signal anti-inflammatory responses mediated by IL-10 in the gut, thus playing a critical role in prevention of excessive Th1 response and chronic inflammation. According to that, in the adult life, Stat3 is required for homeostasis and regeneration of the adult intestinal epithelium under stress conditions (Phesse et al., 2014). On the other hand, STAT3 is also the major protumorigenic IL-6 effector: STAT3 ablation in intestinal epithelial cells interferes with tumor formation and tumor growth in a mouse model of Colitis Associated Cancer (Grivennikov et al., 2010).

In our *Tg(7xStat3-Hsv.Ul23:EGFP)* reporter, a strong EGFP expression reveals Stat3 pathway activity in isolated cells of the normal adult zebrafish intestine, meaning that Stat3 presence in the intestine is not always linked to pathologies. Our results show that Stat3 pathway is active in a restricted population of intestinal cells, identified as a stem Columnar Base Crypt cell, located at the base of the intestinal inter-villus pocket.

Consistently with our expectations, Stat3 intestinal stem cells are actively proliferating, which is in agreement with the actual knowledge on adult stem cells belonging to tissues with a high rate of cell turnover: zebrafish adults replaces the whole intestinal cell population every 7 days.

Interestingly, the same Stat3 positive cells are enriched in number during intestinal cancer formation and progression, maintaining the same proliferative nature they had in physiological conditions. Although the nature and origin of Cancer Stem Cells is nowadays an open research field, one of the theories associates adult stem cells (ASC) with tumor formation. This happens most often with tissues with a short time of cell renewal, such as the skin or the gut, where ASCs are CSC candidates because of their high mitotic index

(compared to most ASCs) together with their long lifespan. This combination creates the ideal set of circumstances for mutations to accumulate and thus for cancer initiation. These hypotheses are also supported by the fact that not every proliferating cell in the tumour context is expressing Stat3, thus excluding the possibility for it to be simply a marker of mitosis in zebrafish. Due to all these findings, we are persuaded Stat3 to be a marker of stemness in the adult zebrafish intestine.

4.4 *Tg(7xStat3-Hsv.Ul23:EGFP)* reporter as a tool to investigate Stat3 during pathogenesis

The investigation of the role of Jak/Stat3 pathway in human disease represents to date one of the most exciting developments in modern medicine (O'Shea et al., 2015).

Most of the major human malignancies manifest elevated levels of constitutively activated STAT3 as well as transcriptional profiles that are consistent with STAT3-regulated gene expression. For many cancers, indeed, elevated levels of activated STAT3 have been associated with a poor prognosis (Johnston and Grandis, 2011). Nevertheless, Stat3 Gain-of-Function mutations were found related to human obesity, various autoimmune disorders including psoriasis, inflammatory syndromes such as Inflammatory Bowel Diseases (IBD) and Neurodegenerative disorders such as Alzheimer's and Huntington's diseases. Finally, loss of function mutations to human *STAT3* causes the Hyperimmunoglobulin E syndrome (HIES or Job's syndrome), a multisystemic disorder that causes recurrent and severe cutaneous and sinopulmonary bacterial infections, connective tissue abnormalities, chronic dermatitis and elevated serum immunoglobulin E (IgE). Because of the plethora of diseases that are caused by Stat3 pathway deregulation, the novel *Tg(7xStat3-Hsv.Ul23:EGFP)* reporter might find several applications when placed in genetic backgrounds that mimics human diseases.

As shown in the results, fluorescence of the *Tg(7xStat3-Hsv.Ul23:EGFP)/apc+/-* fish can be used to follow the dynamics of ectopic activation or down-regulation of the Stat3 pathway during different phases of pathogenic processes. Importantly, the *Tg(7xStat3-Hsv.Ul23:EGFP)* signal can be used to label precisely the identity of specific cells implicated in Stat3-dependent pathologies.

It is important to say that although the reporter physiologically marks only restricted tissues of the nervous system and the gut, the reporter signal becomes detectable under

stress conditions (mechanical damages or bacterial infections), in many other tissues including the muscle, the fin and the adult brain.

4.5 *Tg(7xStat3-Hsv.Ul23:EGFP)* reporter as a tool for drug screening

Although increased levels of phosphorylated STAT3 have been detected in the majority of human cancers and tumor-derived cell lines, STAT3 deregulation is not only a cancer issue. The Jak/STAT3 pathway plays also an important role in various autoimmune disorders, obesity and inflammatory syndromes including Inflammatory Bowel Diseases (IBD) and Neurodegenerative disorders such as Alzheimer's and Huntington's diseases.

Interestingly, although Stat3 KO in mice is embryonic lethal, cumulative data obtained from conditional knockouts indicates that STAT3 may be dispensable for the function of normal cells and tissues (Frank, 2007), suggesting that potential drug-based therapies targeting STAT3 might be well tolerated. However, despite this potential as a therapeutic target, and the extensive attempts by many laboratories and pharmaceutical companies to develop an effective STAT3 inhibitor for use in the clinic, no direct STAT3 inhibitor has been approved for clinical use. (Wake and Watson, 2015).

Nevertheless, there are multiple potential upstream inputs that lead to the activation of STAT3, including: the human epidermal growth factor receptor (EGFR), the family of IL-6–type (IL-6) cytokine receptors that form complexes with gp130 and JAKS, and several GPCRs (Johnston and Grandis, 2011). Consequently, targeting these kinases has valuable potential for STAT3 inhibition. To date, numerous small molecule inhibitors have been developed targeting Stat3 upstream regulators, some of which are currently undergoing various stages of clinical trials for chemotherapy as well as for treatment of inflammatory syndromes such as rheumatoid arthritis, psoriasis, and IBD (Kim et al., 2016).

With this work we suggest the *Tg(7xStat3-Hsv.Ul23:EGFP)* reporter as a novel tool for drug screenings targeted to Stat3 or to Stat3 upstream positive regulators. Our results demonstrate that administration of Stat3 pathway inhibitors to the embryos, is faithfully reported by a significant decrease in the fluorescent signal. In medical research, specifically, this represents the opportunity to test novel Stat3-targeted molecules directly by dissolution of them into the embryos medium or by injection of the drugs into

the vascular tissue of the larvae. This allows rapid screenings of many different molecules, simply by *in vivo Tg(7xStat3-Hsv.Ul23:EGFP)* fluorescence observations.

In our zebrafish model for intestinal colorectal cancer, the *Tg(7xStat3-Hsv.Ul23:EGFP)* reporter signal was significantly enhanced in the tumour mass, with respect to the normal tissue. This result reveals the possibility to perform novel chemotherapy screenings for Stat3-targeted drugs, and to specifically follow *in vivo* the effect of the drug on a solid tumour by simple epifluorescence microscopy without need to sacrifice the sample.

Nevertheless, xenotransplantation of cancer cells from *Tg(7xStat3-Hsv.Ul23:EGFP)* hyperplasic tissues onto WT larvae, followed by screening of anti-Stat3 drugs by direct observation of cancer cells with epifluorescence microscopy, might be a valid and more ethical alternative to the test on adult fish.

4.6 Stat3 regulates proliferation through mitochondrial DNA transcription

Stat3 has been studied extensively as a nuclear transcription factor, however the finding that Stat3 also localizes to mitochondria opened a new area of research.

The first evidences reporting the existence of a pool of Stat3 that localizes to mitochondria, which was named mitoStat3, were collected by Wegrzin et al. in 2009. In that publication they identified Stat3 as a positive regulator of mitochondrial electron transport chain (ETC), being incorporated in the inner mitochondrial membrane together with GRIM-19 protein, a subunit of complex I.

Moreover, they specifically exclude a transcriptional role for mitoStat3 and instead purpose a mechanism of direct interaction of Stat3 with complexes I and II of the ETC, whose activity is disrupted in Stat3-/- cells. On the other hand, more recent publications showed the direct binding of Stat3 protein on mtDNA in keratinocytes culture (Macias et al, 2014) and positive mitoStat3-dependent transcriptional regulation of mitochondrial genes, able to promote mouse ESC proliferation and induce naïve pluripotency (Carbognin et al., 2016).

In this work we showed *in vivo* the dependence on Stat3 mitochondrial transcriptional activity of stem cells proliferation, thus corroborating the hypothesis of a dual nuclear and mitochondrial transcriptional function for Stat3 protein.

In zebrafish injection of murine MLS_Stat3_NES at 1-2 cell stage strongly activates mtDNA transcription at 48 hpf, specifically in the OT region containing the proliferating stem niche. Coherently with that, the mitosis rate in that same tissue is much enhanced after injection of MLS_Stat3_NES, supporting the in vitro results of Carbognin et al.. To exclude the possibility that mitoStat3 promoted proliferation by its acitvity on Complex I and II, we injected the same MLS_Stat3_NES while blocking mtRNA Polymerase. Our experiment showed no significant increase in the proliferation rate of OT stem niche cells, confirming that mitoStat3 regulates embryonic stem cell proliferation *in vivo* by mtDNA transcription.
We also investigated the relevance of two post translational modifications (p-Y705, p-S727) in mitoStat3-driven gene transcription, by injecting two mutated forms of murine Stat3 (Y705F, S727A) into 1 cell stage embryos. Both mutated forms did not show any increase in mtRNAs expression. This result suggests that both sites must be phosphorylated for Stat3 to exert its mitochondrial transcriptional activity. Moreover, chemical inhibition of endogenous Stat3 Y705 phosphorylation was also associated with reduced mtRNA expression and inhibition of proliferation in the nervous system at 72 hpf, thus confirming Y705 phosphorylation to be indispensable for mitoStat3 functions.
In their 2009 work, Wegrzin et al., also investigated the relevance of p-Y705 and p-S727 in the Stat3 mitochondrial functions, specifically focusing on the rescue of complex I and II activity into STAT3-/- cells. Unlike ours, their results suggested a dispensable role of p-Y705 for mitochondrial ETC functionality, which to them was only dependent on S727 phosphorylation. Anyway, their experimental procedures were performed using murine Stat3 forms containing the mentioned MLS, which is not coded in the Stat3 protein sequence; although the mechanism of Stat3 import into mitochondria remains obscure, *in silico* studies showed that the protein does not have a canonical mito-targeting sequence. One could argue, based on this knowledge, that the necessity of Y705 phosphorylation for Stat3 import into mitochondria, might be bypassed by the presence of the MLS, thus justifying the discrepancy between our results and those obtained by Wegrzin et al.. Nevertheless, many publications reported that both Tyr705 and Ser727 phosphorylation of Stat3 are present in mitochondria (Wegrzin et al., 2009; Zhang et al, 2013). On the other hand, our results suggest a tissue specific activation of mitoStat3-driven transcription, that only resides in proliferating stem cells, according with Carbognin et al. findings. Considering that, mitoStat3 role of transcriptional regulator and proliferation promoter might be specific for ESCs, thus being impossible to visualize in differentiated mouse and human cell lines.

To conclude, it is important to notice that the tissues where the transcriptional activity of mitoStat3 is more relevant are the same where the *Tg(7xStat3-Hsv.Ul23:EGFP)* line, which is only reporting nuclear Stat3 activity, is more active at 48 and 72 hpf. In our opinion, an explanation to that, might reside in the dependence of Stat3 nuclear and mitochondrial transcriptional activity on post-translational modifications. Previous evidences reported the insufficiency of Stat3-C constitutively dimeric form to restore ETC activity in Stat3 deficient cells, postulating then the necessity of monomeric Stat3 to bind complex I (Wegrzin et al., 2009). On the other hand, Stat3 nuclear and mitoStat3 transcriptional activities are targeted towards specific Stat3 responsive elements, that are highly conserved between genomic and mitochondrial DNA transcriptional promoters: examination of mouse and human mtDNA sequence revealed multiple generic Stat3 (TTN_4AA) binding sequences within the regulatory d-loop region (Macias et al., 2014). Moreover, to be able to bind to its DNA responsive elements Stat3 must be in the dimer form. Due to this knowledge, one could speculate on the possibility for Stat3 to exert its transcriptional activity within the mitochondrion only in its dimeric form and thus only when phosphorylated to its Y705. Accordingly, nuclear and mitochondrial Stat3 transcriptional activities might both rely on Y705 phosphorylation and subsequent Stat3 dimerization, thus occurring together within the same cell.

If true, this topic might explain the discrepancy in the results obtained by us with respect to the previous results by Wegrzin et al., and moreover would represent the possibility for the *Tg(7xStat3-Hsv.Ul23:EGFP)* reporter to be used as a bona fide tracer of cells with Stat3-driven mtDNA transcription.

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