Regulation of Notch signaling in the heart by epigenetic modifications

PhD thesis in Molecular Genetics and Biotechnologies

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Scuola Normale Superiore

2015

INDEX

SYNOPSIS

Understanding the molecular mechanisms regulating cardiac cell proliferation during the embryonic, fetal and adult life is of paramount importance in view of developing innovative strategies aimed at inducing myocardial regeneration after cardiac damage.

The Notch pathway plays a key role in the regulation of cardiomyocyte proliferation during mammalian embryonic life. Moreover, it is essentially involved in the cardiac regeneration process after injury in Zebrafish. Therefore, we assessed the efficacy of Notch pathway activation to sustain cardiac regeneration in a model of myocardial infarction in mice.

During early postnatal life, cardiomyocytes exit the cell cycle. We demonstrated that this event is paralleled by a decrease of Notch signaling and by the establishment of a repressive chromatin environment at Notch target genes, characterized by Polycomb Group protein 2-mediated silencing. The stimulation of the Notch pathway through Adeno-associated virus-mediated gene transfer of activated Notch1 or of the soluble form of the ligand Jagged1 prolonged the capacity of cardiomyocytes to replicate, which correlated with an increased rate of Notch target gene expression and the maintenance of an open chromatin conformation at Notch target gene promoters. However, the same vectors were ineffective in stimulating cardiac repair in a model of myocardial infarction in adult mice, despite efficient transgene expression. We identified the molecular cause of the lack of action of Notch signaling stimulation in adults in the increased DNA methylation at Notch target gene promoters, which correlated with permanent switch off of the Notch pathway.

Our results confirm that the Notch pathway is an important regulator of neonata adults, due to the permanent epigenetic modifications at the DNA level at Notch responsive genes l

INTRODUCTION

HEART DEVELOPMENT AND FUNCTION

The heart is the first organ to form and to become functional in the embryo during its development. It is a complex organ composed of different muscle and non-muscle cell types (**Fig.1**): atrial/ventricular cardiac myocytes, conduction system cells, smooth muscle/endothelial cells of the coronary arteries and veins, endocardial cells, valve components and connective tissue. The major sources of precursor cells during heart development have been identified in cardiogenic mesoderm, cardiac neural crest and the proepicardial region.

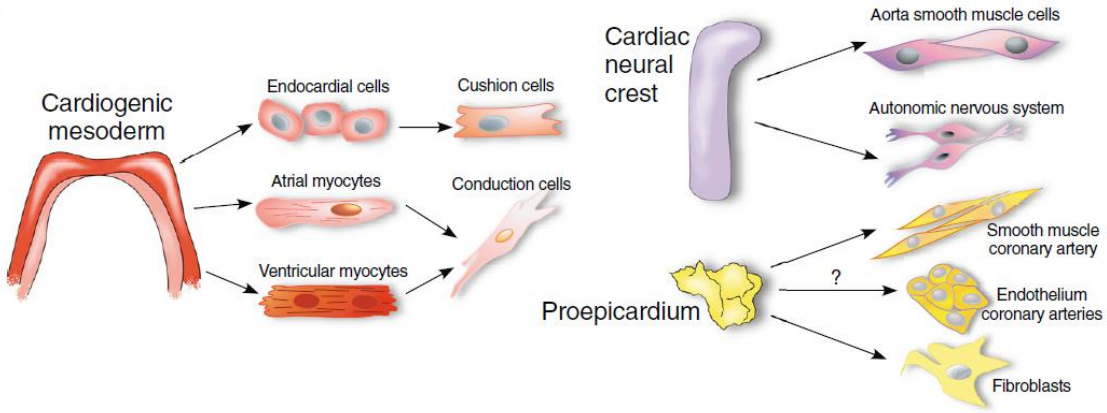


Figure 1. Source of the different cellular components of the heart (adapted from [1]).

The genetic program responsible for heart development is evolutionarily conserved and is driven by a complex network of signaling molecules and tissue specific transcription factors, which control the activation of the genes responsible for heart morphogenesis (reviewed in [2-4]). Cardiac myocyte progenitor cells are already identifiable in the late gastrulation phase as an epithelial cell population in the

cranio-lateral mesoderm, the primary heart field, which gives rise to the cardiac crescent. The commitment of mesodermal cells to a cardiogenic fate strictly depends on the paracrine signaling between the endoderm, which secretes bone morphogenetic proteins (BMPs), positive regulators towards the cardiac lineage and the ectoderm, which secretes Wnt inhibitors [5-6]. The ultimate response to these molecules triggers the expression of specific sets of cardiogenic genes, which drive the morphogenetic events involved in heart development. In Drosophila, pro-cardiogenic signaling involves the transcription of the homeobox gene *Tinman*, necessary to activate the transcription of *Mef2*, which controls the differentiation of the precursors toward cardiomyocytes [7]. In vertebrates, Nkx2.5, the orthologue of Tinman, is expressed very early; its expression is cardio-specific and is maintained throughout the life. However, the factor does not appear to be necessary at this stage for cardiomyocyte specification, while mutant Nkx2.5 embryos die later during development due to abnormality in heart tube morphogenesis and left ventricle development [8]. Tinman/Nkx2.5 cooperates with the Gata family of transcription factors, important gene regulators of the cardiac developmental program. The cardiac crescent becomes organized as the linear heart tube, consisting of an inner layer of endocardial cells and an outer layer of myocytes held together by a dense extracellular matrix known as cardiac jelly. The linear heart acquires a rightward spiral form through a looping process, tightly controlled by an asymmetric axial signaling system. At this point, the cell fate of the precursors of the four cardiac chambers is already genetically determined.

Even if the genetic circuitry involved is not completely defined yet, the basic helix-loop-helix transcription factors Hand1 and Hand2 have been identified as important regulators of the development of the left and right ventricular segment respectively [2, 4]. At this stage, a second population of cardiac progenitors of splanchnic mesoderm is recruited; interestingly it seems that the main difference between the primary and the secondary heart field progenitor population is the timing of differentiation and not the expression pattern, since they both express similar

transcription factors [9]. These so-called secondary heart field progenitor cells contribute to right ventricle, atria and outflow tract formation. Newly formed cardiomyocytes start to secrete the extracellular matrix, resulting in the formation of the cardiac cushions, rapidly colonized by endocardial cells. Subsequently, a massive wave of myocardial proliferation triggers the formation of cardiac trabecolae.

The growth of each chamber results from a combinatorial signaling between cell layers. The endocardium secretes Neuregulins, the receptors of which are expressed by the myocardial layer; this signaling pathway is involved in the growth process of the developing ventricles [10]. At this stage, swelling of the cardiac cushion separates the cardiac tube into distinct chambers, causing septation.

Precursors of the cardiac valves arise from cardiac cushions: cells of endocardial origin migrate into the cardiac cushions and differentiate into fibrous tissue, responding to a complex signaling network, regulated at some extent by TGFβ family members [2, 11]. Other precursor cell populations migrate at this point to different areas of the developing heart to contribute to its final features: cardiac neural crest cells give rise to the vascular smooth muscle of the aortic arch and great vessels; epicardial cells, derived from the proepicardium, progressively envelop the developing heart; coronary precursor cells contribute to the coronary vasculature. From the epicardium, through epithelial to mesenchymal transition, a population of mesenchymal cells also arises, which contributes to the development of connective tissue, fibroblasts and smooth muscle cells of cardiac vessels [1].

Finally, the conduction system of the heart is essentially of myocardial origin: the decision of becoming conducting myocytes is regulated at the genetic level through the expression of a peculiar transcriptional network, while the cardiac ganglia innervating the conduction system are mainly derived from the neural crest [12]. The epicardium contributes to the conduction system through interstitial fibroblasts sparsely found between the mature cells of the conduction system (scheme of heart development in **Fig.2**).

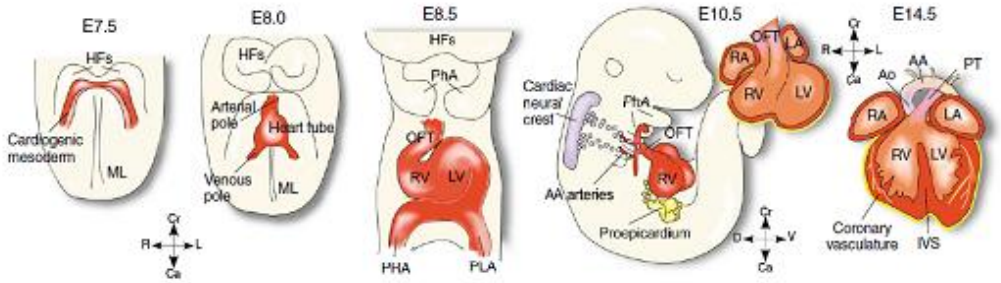


Figure 2. Schematic representation of heart development (adapted from [1]).

The structure of the mature heart (**Fig.3**) reflects its embryonic development as a muscular tube. The right atrium receives venous blood, which enters the right ventricle through the tricuspid valve. From there, the blood is then pumped by the ventricle through the pulmonary artery to the lungs, where it is oxygenated; the oxygenated blood returns to the heart in the left atrium through the pulmonary veins and then it passes into the left ventricle through the mitral valve, from where it is pumped in the arterial vascular circuit in the body. Every heartbeat originates at the sino-atrial node located at the junction between right atrium and superior vena cava. The electrical impulse is propagated through the atria to the atrioventricular node and from there to the ventricle.

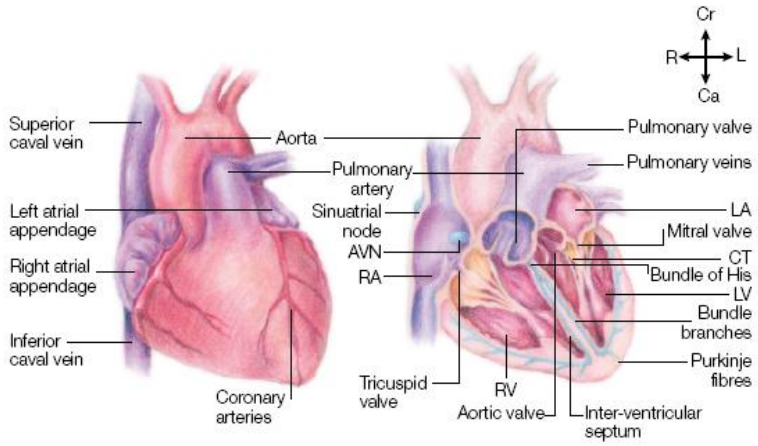


Figure 3. Adult heart structure (adapted from [3]).

IS THE HEART A POSTMITOTIC ORGAN?

During the early postnatal life, a switch takes place between myocyte hyperplasia and hypertrophy. In humans, after withdrawal from the cell cycle, there is almost a threefold increase in the diameter of cardiomyocytes [13-14]. The paradigm of the heart as a postmitotic organ was established in the 1950s, when the first studies regarding heart growth were published [15-16]. First detection of mitotic figures in adult cardiomyocytes dates long back [17]. In more recent years, evidences of adult cardiomyocyte proliferation in human and rodent samples were obtained, showing that the mammalian heart maintains a mitotic activity even in adult organisms, where DNA duplication [18] and metaphasic chromosomes were detected in heart sections [19]. The detected proliferation rate was very variable in different studies, but consistently very low [20-21]. Recently, the attempt to precisely quantify cardiomyocyte turnover gave very different results according to the method used: using ^{14}C dating, the Frisen group reported that cardiomyocytes are renewed with a gradual decrease from 1% turn over annually at the age of 25 to 0.45% at the age of 75, therefore confirming a low proliferation rate [22]. A 20 fold higher turnover rate was calculated analyzing the incorporation of labeled nucleotides in cardiomyocytes [23], pointing to an underestimation of the number of proliferative myocytes in the previously reported low proliferation rates. Actually, the reported rate of proliferation by Bergmann and colleagues is in agreement with the known range of ploidy in human cardiomyocytes [24] and is consistent with the lack of significant regeneration after damage in the adult hearts. A variety of studies have identified proliferative cardiomyocytes dividing symmetrically throughout all the life span, albeit at a very low level; it has been proposed that the low turn-over due to adult cardiomyocyte proliferation is an important mechanism to maintain myocardial homeostasis [25-27].

The results are quite debatable also in animal models: in rodents, the analysis of a variety of mitotic markers in embryonic, neonatal, and adult phase has demonstrated robust cardiomyocyte proliferation in embryonic life only, followed mainly by binucleation of myocytes in the perinatal period, superimposable to their exit from the cell cycle [28]. The idea of a non proliferative adult heart is in agreement with the results of a recent study by Porrello and colleagues, who reported complete myocardial regeneration achieved through formation of new contractile cardiomyocytes in case of apical resection of the heart in 1-day old mice, while the formation of a fibrotic scar was prevailing when the ventricular resection was performed in 7-days old mice [29]. This evidence strongly supports the conclusion that the mammalian heart is endowed with an endogenous regenerative potential in fetal and early neonatal life only.

Discrepant results have been published regarding cardiomyocyte proliferation in the case of myocardial injury: Hsieh and colleagues identified an increased rate of cardiomyocyte proliferation after myocardial infarction in mice, possibly due to precursor cell proliferation [25]. A more recent study however indicated that the role of cardiac progenitors after injury is very limited [26]. The topic is really highly debated: besides the identification of the population (stem cells or differentiated cardiomyocytes) able to proliferate and eventually repair the heart in case of injury, the concept itself of the existence of a regenerative pathway in mammals still remains highly controversial. A recent study which did not detect any increase in the basal proliferation rate in case of myocardial infarction in mice have added further fuel to the controversy, even questioning the efforts to trigger any myocardial regeneration in mammals [27]. Finally, recent results by Naqvi and colleagues have also reopened the discussion on the proliferative potential of the postnatal heart in mammals, by showing that a second window of transient cardiomyocyte proliferation occurs post-natally in preadolescent (P15) mouse hearts, which appears to be regulated through the IGF-1/Akt pathway, known to modulate the early postnatal stages of heart development [30].

Although there are contrasting evidences about adult cardiomyocytes proliferation, what remains unquestionable is that the mammalian heart is unable to recover after massive cardiomyocyte loss, as in the case of myocardial infarction. Since cardiovascular diseases are the leading cause of mortality worldwide, among which ischemic heart disease is the most frequent disease condition, the strong need for therapies triggering cardiac regeneration still remains completely unmet.

CARDIAC REGENERATION IN MAMMALS

As depicted in **Fig.4**, cardiac regeneration has been approached in a variety of manners, from endogenous stem/progenitor cells proliferation stimulation to exogenous cell therapy, from stimulation of resident adult cardiomyocyte proliferation to prevention of cardiomyocyte apoptosis.

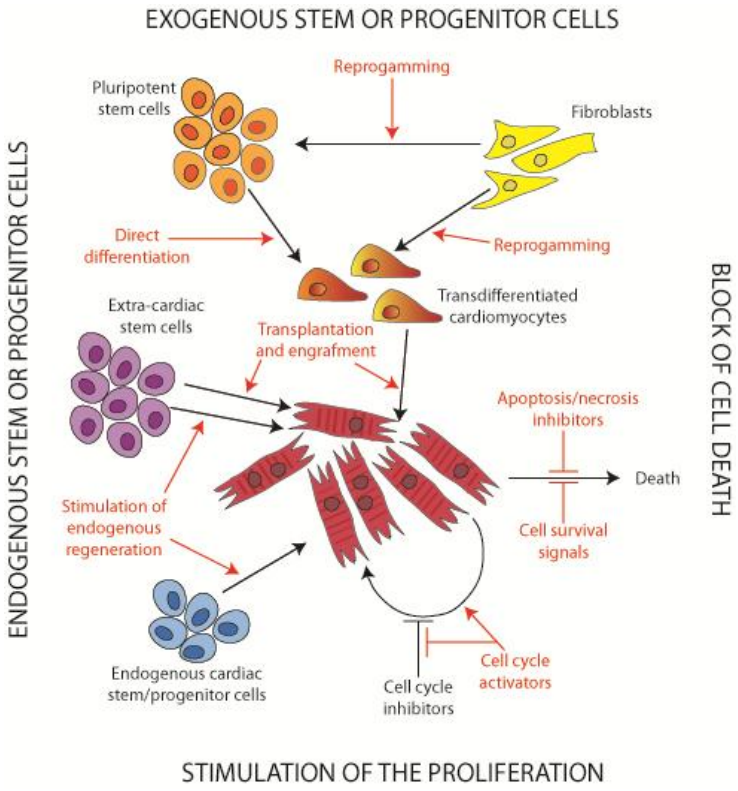


Figure 4. Different strategies to stimulate cardiac regeneration.

Antagonizing cell death and enhancing survival pathways in cardiomyocytes after an ischemic event are strategies which are feasible, but showing one major constraint: they are useful to prevent cell death, but cannot reconstitute cardiac muscle cell loss, which is one of the unsolved problems occurring after myocardial infarction [31]. Therefore major attention has been given to strategies which are aimed at reconstituting the cardiomyocyte population.

Accumulating evidences on adult cardiomyocyte division suggest that one of the main approaches to stimulate regeneration is to increase the number of dividing pre-existing cardiomyocytes. This task has been pursued manipulating different key players of the cell cycle or developmental signals known to act on cardiomyocytes. These include stimulation of Cyclin D2 [32] or Cyclin A1 [33], inhibition of p38 [34-35], stimulation of Periostin downstream signaling [36] or Neuregulin1-mediated ERBB2 pathway activation [37]. In vivo, some of these strategies have led to better functional outcomes in animal models, but the efficiency in promoting adult cardiomyocyte proliferation has been generally very limited, with a small number of mainly mononucleated adult cardiomyocytes completing cell division. Recently, miRNAs have been demonstrated to be an efficient, novel tool to achieve cardiomyocyte proliferation. Inhibition of the miR-15 family, which is involved in cell cycle arrest, has been shown in vivo to prompt adult cardiomyocytes to proliferate and improve functional outcome after myocardial infarction [38], while administration of miR-199a-3p and miR-590-3p were demonstrated to trigger cardiac regeneration, improving functional outcome after infarction and stimulating adult cardiomyocyte proliferation [39].

An alternative strategy to be considered to pursue heart regeneration is the formation of new myocytes from multipotent cells, resident progenitor cells committed to cardiac phenotype, stem cells present in the niches, or ex vivo transplanted cells.

Stem cells and progenitor cells

Historically, among adult stem cells, bone marrow derived cells (BMC) were the first population of stem cells reported to possess regenerative capacity in vivo. Orlic and collegues identified bone marrow cells able to transdifferentiate into cardiomyocytes and vessels once injected into the infarcted myocardium, providing significant functional benefit [40]. Similar results were concomitantly reported in other studies [41-42]. These results, however, have later been heavily questioned by a vast part of the scientific community [43-44]. Given the initial enthusiasm in the field, several clinical trials in patients with acute myocardial infarction or ischemic heart disease have been performed; transplantation of different subtypes of bone marrow-derived cells resulted in very different outcomes. The first clinical trial in which autologous mononuclear bone marrow cells were transplanted into the infarcted region claimed that the treated patients had better functional heart parameters due to the BMC-associated myocardial regeneration and neovascularization [45]. The subsequent clinical trials generated contrasting results, showing no significant improvement of heart function in the patients infused with autologous mononuclear bone marrow cells [46]. Several other trials have been performed later [47], and their results were collectively analyzed in 2012, reporting a modest but significant improvement of the left ventricular ejection fraction in the treated patients, together with a small beneficial effect in other left ventricular parameters [48]. More recently, other two clinical trials were performed, both reporting lack of effect of bone marrow mononuclear cell delivery [49-50].

Taking these evidences together, considering the differences in injection system, bone marrow cell preparation and evaluation of functional parameters, the overall result is that the injection of the cells is safe and feasible, even though the beneficial effect is very modest, possibly due to a paracrine secretion of angiogenic or pro-survival factors, which could stimulate cardiomyocyte survival, or perhaps resident stem cells proliferation, therefore exerting a beneficial action of potential interest for the development of cell-free treatment [51].

Many different groups have described the existence of a pool of self-renewing, cardiac resident progenitor cells (CPCs) There is no unanimous agreement on the markers of this population: overlapping populations expressing Sca-1 [52], c-kit [53] or Abcg2 [54] have been described able to differentiate towards cardiomyocytes in vitro. One strategy to exploit this population for therapeutic approaches is its in vitro expansion. Studies on the c-kit+ population, which was reported to be present in humans, gave rise to controversial results: in an initial study, c-kit+ cells, isolated, expanded in vitro, differentiated toward cardiomyocytes, were transplanted in the context of myocardial infarction in rodents and were reported to support regeneration forming news cardiomyocytes [53, 55] or acting through a paracrine effect [56]. In other studies no transdifferentiation of c-kit+ positive cells to cardiomyocytes was detected [57-58]. The SCIPIO phase I clinical trial was performed injecting autologous CPCs in patients with ischemic cardiomyopathy [59-60]; the results seemed encouraging, suggesting that intracoronary infusion of these cells improved heart functional parameters in patients, therefore suggesting to proceed to a phase II study. Recently, however, the phase I study results have been questioned [61].

Another population of progenitor cells are the cardiosphere-derived cells, described as a population of stem cells isolated from biopsies, which can be expanded in vitro [62]. These cells were reported to exert beneficial effects when transplanted after myocardial infarction, thanks to their differentiation to new cardiomyocytes and to the secretion of factors exerting a beneficial paracrine effect [63]. Also in this case, there is controversy on the cardiomyogenic potential of these cells. One report has not confirmed their capacity to differentiate into cardiomyocytes and has proposed a fibroblast origin for these cells [64], while more recent data have indicated that their beneficial activity has essentially to be ascribed to their paracrine action [65]. Despite these controversies, cardiosphere-derived cells have been used for a clinical trial in patients with myocardial infarction, with reported beneficial results [66].

Pluripotent stem cells

Many attempts have also been performed to differentiate embryonic stem cells (ESC) and, more recently, reprogrammed induced pluripotent stem cells (iPS) to cardiomyocytes. The latter cell type is particularly appealing for regenerative purposes, since ESC-based therapies would be allogeneic, therefore requiring immunosuppression, while iPS cells would allow autologous transplantations. Several groups have successfully differentiated these cells into cardiomyocytes exhibiting intrinsic contractile activity and expressing cardiac transcription factors, but with myofibrillar organization typical of early-stage cardiomyocytes, therefore resembling immature cells [67-70].

In the context of an injured heart, ESC-derived cardiomyocytes were demonstrated to differentiate into immature cardiomyocytes, regenerate infarcted myocardium and achieve electromechanical integration with the surrounding tissue [71-73]. Not all the studies are concordant on the long term effect of the transplantation: it was also reported that, even in the case of graft survival, at longer time points the beneficial effect was not maintained [74]. Recent work by Murry and colleagues addressed the effect of human ESC-derived cardiomyocyte (hESC-CM) grafts in larger animal models. Non-human primates underwent myocardial infarction, followed by the injection of hESC-CMs. This study demonstrated for the first time the feasibility of the large-scale production of hESC-derived cardiomyocytes. The functional effect of the graft was evaluated, demonstrating that human derived cells provide re-muscularization to the infracted heart, showing electromechanical coupling with the host cardiomyocytes and perfusion by the host vasculature. The study revealed, however, the onset of nonfatal ventricular arrhythmias in the grafted primates, pointing to the need to deeper understanding the phenomenon, in order to achieve safe clinical translation to patients [75].

As far as iPS-transplantation is concerned, this now appears as a very promising avenue for the generation of cells capable of regeneration of various organs and tissues, including the heart. Indeed, pioneering studies have also indicated that iPS-

derived cardiomyocytes can engraft in the infarcted heart and provide therapeutic benefic in small animal models [76]. A relevant fear that might hamper further development of the iPS technology is the possibility that immature, non-differentiated iPS cells might give rise to the formation of teratomas. Indeed, a case of teratoma induced by the transplanted cells in the context of myocardial infarction, probably caused by incomplete differentiation of the cells, has been already reported [77].

A final interesting approach to achieve therapeutic formation of novel cardiomyocytes is the direct reprogramming of fibroblasts to become cardiomyocytes. A first attempt was performed to differentiate fibroblasts into cardiomyocytes using the iPS technology with a cocktail of 14 different transcription factors, out of which three (Gata4, Mef2C, c-Myc) were necessary to achieve the reprogramming of mouse cardiac fibroblasts, showing gene expression shifting from a fibroblast- to a cardiomyocyte-like profile. Even if the percentage of fully reprogrammed cells was around 1%, the reprogrammed fibroblasts were able to differentiate into cardiomyocytes when transplanted into mouse hearts [78].

Other strategies have been further applied to optimize the reprogramming: the so-called "Yamanaka cocktail" of genes (Oct4, Sox2, Klf4, c-Myc) and the addition of cardiogenic factor BMP4 were demonstrated to have a higher efficiency in converting mouse embryonic fibroblasts to cardiomyocytes [79]. A few studies also reported direct reprogramming in vivo. Using genetic lineage tracing experiments, resident non-myocytes were demonstrated to be reprogrammed into cardiomyocyte-like cells by in vivo local delivery of the 3 factors described by Ieda (Gata4, Mef2c and Tbx5), triggering also decreased infarct size and slightly better functional outcome in infarcted mice [80]. The authors believe that the in vivo administration of the reprogramming factors, which results in functional better outcome in the case of myocardial infarction, could be due to the higher efficiency of reprogramming achieved in the heart environment, compared to a Petri dish. The authors also hypothesized that some mechanisms, such as cardiac fibroblast activation block,

enhanced survival of cardiomyocytes, facilitated differentiation of cardiac progenitors or improved angiogenesis could contribute to the benefits observed upon expression of the reprogramming factors in the heart after myocardial infarction [81]. Finally, the reprogramming of cardiac fibroblasts to cardiomyocytes, both in vitro and directly in vivo, was also achieved using a combination of four microRNAs, which were able to induce direct cellular reprogramming in vitro, while the administration of the same microRNAs in vivo into the ischemic mouse heart results in the conversion of cardiac fibroblasts to cardiomyocytes [82]. Further improvement in the reprogramming process appears to be still needed, since often the reprogrammed cardiomyocytes show an immature phenotype [83] and the efficiency of the process is still remarkably low.

First attempts at reprogramming human fibroblasts were recently made by combining various cardiac transcription factors and/or microRNAs: the reprogrammed fibroblasts showed some sarcomere-like structures, calcium transients and a cardiomyocyte-like gene expression profile [84-85].

Epicardial stem cells

Considering the fundamental role of the epicardium during heart development and the variety of cell types that origin from this layer, epicardial progenitor cells (EPDC) in the context of heart injury have also been investigated. In the adult mouse heart, the epicardium overlaying the infarcted area is locally disrupted. In response to the injury, the surrounding epicardium undergoes a transient reactivation of the embryonic gene program [86]. The epicardium overlaying the infarcted areas is therefore regenerated in 3 days after the injury. This process is also paralleled by the formation of a thick layer of subepicardial mesenchyme above the infarcted area, originated by epicardial cells undergoing epithelial-mesenchymal transition (EMT), which contribute predominantly to fibroblasts, to a lesser extent to the coronary vasculature and possibly to cardiomyocytes [86]. This evidence demonstrates that a regenerative response is started, but is not effective. Recently,

it has also been reported that epicardium-derived cell activation, stimulated by myocardial injury, could substantially contribute to repair of the adult heart via regeneration of the coronary vasculature and of the myocardium vasculogenesis in the presence of Thymosin β4 (Tβ4) [87]; moreover Tβ4-activated EPDCs were reported to transdifferentiate into new cardiomyocytes, structurally and functionally integrated with the resident muscle, when the Tβ4 priming was performed prior to myocardial infarction [88], while no effect on EPDCs was detected when Tβ4 was administered after the injury, where EPDCs were contributing mainly to fibroblast population [89-90]. Finally, these cells were also reported to contribute to the reduction of infarct size through stimulation of angiogenesis and secretion of paracrine factors which can modulate the subepicardium compartment [91]. A deeper understanding of the potential of epicardial population is needed in order to evaluate the therapeutic potential of these cells in regenerative medicine.

CARDIAC REGENERATION IN ZEBRAFISH

It is widely known that organ regeneration can efficiently take place in lower vertebrates. In particular, Zebrafish has a high regenerative capacity since in adults amputated or injured tissues such as fins, maxillary barbel, retina, optic nerve, spinal cord, brain, pancreas, kidney and heart muscle can regrow [92].

The resection of up to 20% of the heart ventricle results in the immediate formation of a clot, replaced in a couple of days by fibrin deposition, followed by the production of new, viable and functional myocardium, reaching perfect recovery 60 days after the heart damage (**Fig.5**) [93]. Three different mechanisms have been proposed to explain which is the cell compartment responsible for the regeneration process: adult, contractile, differentiated myocytes could be stimulated to massively re-enter the cell cycle and reform the apex; regeneration could proceed through the recruitment of undifferentiated progenitor cells which are differentiating into new

cardiomyocytes; pre-existing cardiomyocytes could undergo "de-differentiation", downregulating contractile genes, in order to create a population of less differentiated cells, able to proliferate and re-differentiate into cardiomyocytes [94]. Initially, it was proposed that regeneration was triggered by progenitor cells in the blastema, which started to express pre-cardiac markers and contractile genes and to proliferate. The authors speculated that injury-related signals are insufficient to stimulate massive adult myocardial cell proliferation, therefore progenitor cells contributed to the regeneration process [95]. This mechanism has recently been disproved by genetic fate mapping experiments, showing that the newly formed cardiomyocytes originate from the adult cells, which undergo partial dedifferentiation, detachment one from another and disassembly of the cytoskeleton, with no reactivation of the fetal gene program [96]. Non myocardial cells play a role in the regeneration process, as they create a suitable environment for myocardial proliferation, expressing a variety of factors, among which Raldh2, a retinoic acid-synthesizing enzyme, shown to be necessary for a correct injury-response to occur [97].

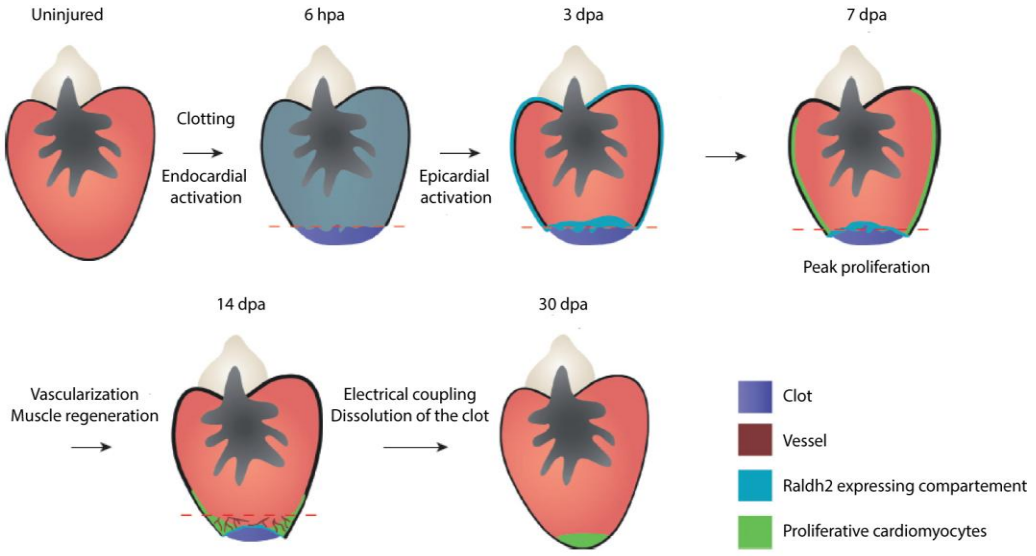


Figure 5. Schematic representation of Zebrafish heart regeneration after ventricular resection [98].

The Notch signaling pathway, through the Zebrafish orthologue Notch1b, was demonstrated to be involved in this heart regeneration process. As in the case of fin regeneration, the expression of Notch1b dramatically increases the day after the amputation and declines two weeks later. The Notch receptor ligand DeltaC parallels the expression pattern of Notch1b. The discovery that the Notch pathway is involved in heart regeneration was particularly interesting, since this pathway is not involved in the genetic program leading to heart development in Zebrafish, pointing out the existence of a specific regenerative genetic program [99]. In a recent study, fate mapping experiments demonstrated that, in the case of ventricular ablation, atrial cardiomyocytes transdifferentiate into ventricular myocytes upon activation of the Notch signaling cascade, since the block of Notch activation impedes the atrial to ventricular transdifferentiation [100]. Recent work has further confirmed the central role of the Notch pathway in the heart regeneration process. Following amputation of the Zebrafish ventricular apex, Notch expression is activated both in the epicardium and in the endocardium and suppression of Notch signaling profoundly impairs cardiac regeneration and induces scar formation at the amputation site; interestingly, the block of Notch signaling in the epicardium and endocardium resulted in decreased proliferation of the cardiomyocyte compartment, where Notch expression was not reactivated upon injury [101]. These results suggest the existence of a complex signaling network downstream Notch activation able to drive the whole regeneration process.

THE NOTCH RECEPTOR PATHWAY

Notch receptors and ligands

In mammals, the Notch receptor family is composed of 4 type-1 transmembrane proteins (Notch1, -2, -3, -4), while there is only one receptor in flies (Notch) and two in *C. elegans* (LIN-12 and GLP-1) (**Fig.6**). The extracellular domain is composed by 29 to 36 epidermal growth factor (EGF) repeats followed by a negative regulatory region (NRR) composed of 3 cysteine-rich Lin12-Notch repeats (LNR) and a heterodimerization (HD) domain. For the interaction with the signal-sending cell, EGF repeats 11-12 are required, while repeats 24-29 prevent the interaction of the receptor with its ligand on the same cell [102-103]. Many of the EGF repeats can bind calcium ions, thus regulating Notch affinity for its ligand and signaling efficiency [104-105]. The LNR motifs and the heterodimerization domain act as negative regulators, preventing receptor activation in the absence of the ligand. The Notch transmembrane domain (TMD) is followed by the intracellular domain, composed by a RAM motif (RBP-Jk association module) involved in the binding of Notch with the transcription factor CSL/RBP-Jk through a high-affinity binding module centered on a conserved WxP motif, followed by an unstructured region containing a nuclear localization signal (NLS), 7 ankiryn repeats involved in protein-protein interaction and an evolutionary divergent transactivation domain (TAD), which recruits transcriptional activators as Mastermind-like and histone acetyltransferase complexes and contains a PEST domain regulating Notch stability and degradation [106-107].

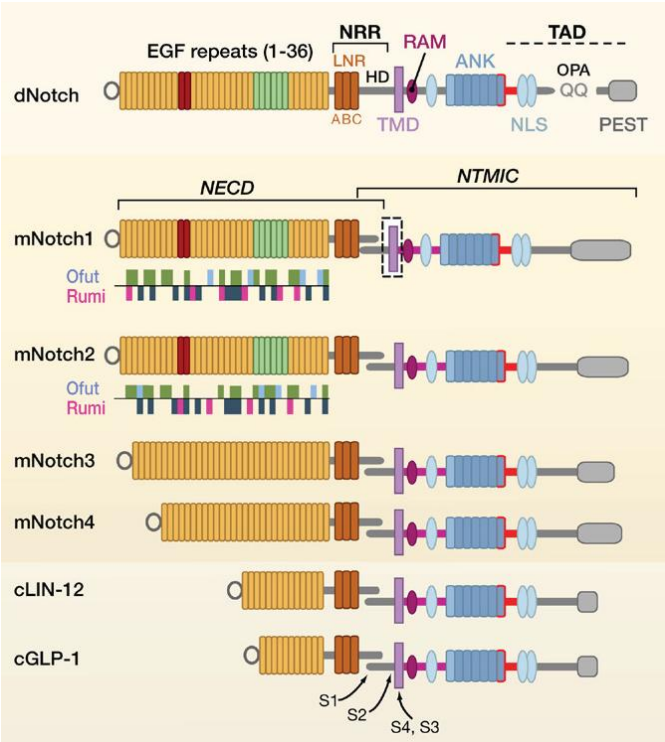


Figure 6. Structure of Notch receptors in flies, mammals and *C. elegans* (adapted from [106]).

There are 5 Notch ligands in mammals: Jagged1 and -2, Delta-like1, -3, -4; in Drosophila they are called Serrate and Delta, and LAG-2 in *C. elegans* (**Fig.7**). They are type-1 transmembrane proteins sharing common features in the extracellular domain, composed by an N-terminal DSL (Delta/Serrate/LAG-2) motif, specialized EGF repeats called DOS (Delta and OSM-11-like proteins) domain involved in receptor binding, and EGF repeats, some of which required for the interaction with the Notch receptors (EGF repeats 11 and 12); only in Jagged1 and -2 there is an additional cysteine-rich region involved in receptor binding specificity. The short intracellular domain is more variable, contains a PDZ domain and is involved in downstream signaling [108].

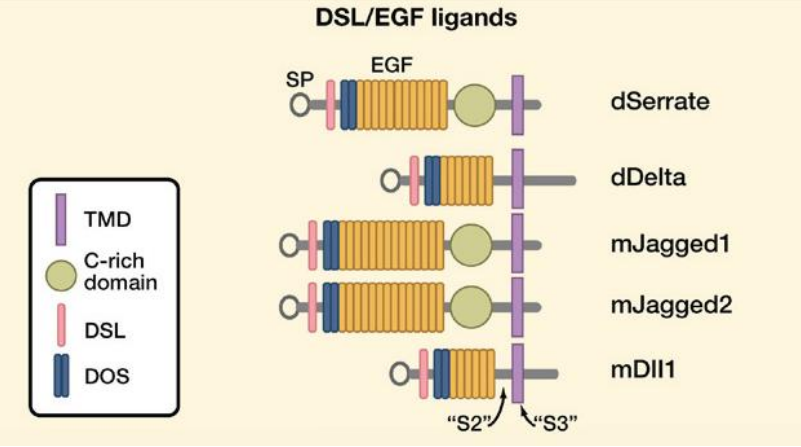


Figure 7. Structure of Notch ligands Jagged/Serrate and Delta in flies and mammals (adapted from [106]).

Notch signaling

Notch protein is synthesized as a peptide of 300 kDa which undergoes glycosylation at different sites of the extracellular domain: the EGF repeats are glycosylated by an O-fucosyltransferase which adds fucose to serine or threonine residues; the O-fucosylation sites can be further modified by N-glycans by the Fringe protein. Fringe glycosylation can affect the ligand-binding activity [109] and could also enhance the cleavage occurring in the Golgi (S1 cleavage), mediated by the Furin-convertase; in mammals, this cleavage leads to a heterodimeric protein with an extracellular subunit of 180 kDa and a transmembrane domain of 120 kDa, non covalently associated. The heterodimer is kept inactive through a tight interaction between the LNRs and the heterodimerization domain. Upon ligand binding, the receptor undergoes a conformational change which results in the exposure of the cleavage site for an ADAM protease (ADAM10, Kuzbanian or TACE, tumor necrosis factor α converting enzyme), 12 amino acids before the transmembrane domain (S2 cleavage) [110]. The cleaved extracellular domain of Notch bound to the ligand is cleared from the membrane through trans-endocytosis into the signal sending cell [111]. The truncated form of Notch resulting from S2 cleavage is the substrate for the subsequent S3 and S4 cleavages operated by γ-secretase, a multicomponent-

intramembrane complex composed by Presenilin, Nicastrin, PEN2 and APH1 [112]. γ-secretase processes Notch at two different sites, eventually leading to the release, into the cytosol, of the Notch intracellular domain (NICD), which is able to migrate to the nucleus [113-114]. In the nucleus, NICD binds CSL (CBF/RBP-Jk in mammals, Su(H) in flies, LAG-1 in *C. elegans*; I will refer to it as RBP-Jk from now onward) transcription factor [115]. In the absence of NICD, RBP-Jk behaves as a transcriptional repressor, binding to histone-deacetylases and other corepressors, keeping the chromatin in a transcriptional silent state [112]. It has been hypothesized that NICD has a stronger affinity to bind RBP-Jk compared to the repressors [116], but this has not been definitively proven. Another model suggests that NICD and the repressors compete for binding to RBP-Jk [116]. After the interaction with NICD, RBP-Jk is converted to an activator of transcription; the complex formed by NICD, RBP-Jk and MAML (Mastermind-like) recruits transcription factors and therefore activates transcription of the target genes, mainly the Hes (Hairy/Enhancer-of-split) and Hey (Hairy/Enhancer-Of-Split Related With YRPW Motif) families of basic helix-loop-helix transcription factors [117-118]. These proteins bind their target sequence on DNA as homo- or heterodimers and act by repressing transcription of their target genes in different ways. The binding of these transcription factors to their cognate binding sites can cause the recruitment of other corepressors, such as Groucho, which can recruit histone deacetylases, therefore causing transcriptional repression through alteration of local chromatin structure; alternatively, they can directly bind to other bHLH factors forming non functional heterodimers [119-120]. Moreover, the NICD-RBP-Jk complex is able to directly activate transcription of several targets, such as Cyclin D1, Cyclin D3, p21, glial fibrillary acidic protein, Myc, Nodal, PTEN, EphrinB2, smooth muscle actin. Interestingly, several of the genes regulated by Notch have a role in the maintenance of a proliferative status [119, 121]. After activation of target gene transcription, NICD is rapidly degraded: MAML couples the activator role with the degradation of NICD, promoting NICD phosphorylation, since the complex composed

of MAML-SKIP-RBP-Jk can recruit the nuclear kinase CycC/CDK8 which hyperphosphorylates Notch TAD and PEST domains [122-123]; the phosphorylated PEST domain is recognized by the ubiquitin-ligase Fbw7/Sel10, resulting in NICD ubiquitin-mediated proteasomal degradation [124-126]. Moreover, acetylation has been demonstrated to regulate the stability of the NICD: in endothelial cells, Sirt1, a member of class III deacetlyases, was discovered to deacetylate and therefore destabilize NICD, triggering its degradation, therefore antagonizing the establishment of the transcriptional activator complex [127].

Recent evidence suggests that endocytosis can modulate Notch trafficking, and therefore its activity. In Drosophila, mutants having defects in endocytosis, recycling, vesicular sorting or multivesicular body formation show defects in Notch signaling [128-129]. A typical way of regulation of receptor internalization is protein monoubiquitination, which can target several residues in the Notch intracellular domain. HECT-type E3 ubiquitin ligase Nedd4 and Itch in mammals (Suppressor of Deltex in Drosophila) can act on NICD as negative regulators. These proteins are involved in a regulatory mechanism to prevent inappropriate ligand-independent activation of Notch signaling, targeting NICD to the lysosomal degradation pathway [130-131]. Besides the involvement of the intracellular trafficking machinery in keeping Notch inactive, endocytosis could also have an active role in NICD release and signaling. Deltex, a ring finger-type ubiquitin ligase, is thought to counteract the effect of Nedd4 and Itch, positively regulating Notch signaling; the factor promotes Notch sorting from the endosomal compartments, escaping the lysosomal degradation [132-133]. Moreover, Notch monoubiquitination followed by endocytsosis is an absolute requirement for γ-secretase to process the receptor. Actually, the initial paradigm of the γ-secretase complex acting on the cell surface has been radically revised, since the multi-subunit protease complex has been visualized embedded in the membranes of the endocytic vesicles, consistent with the evidence showing that it has an optimal activity at a low pH, as it is in the endocytic compartment [134]. These mechanisms of Notch trafficking could have a role in

protecting the cell from accidental firing of the Notch pathway: since very little amount of NICD is required to activate the downstream pathway, this could be generated in a ligand independent manner; the continuous ubiquitination and degradation of the receptors at the cell surface could be a way to regulate the steady-state level of the Notch protein [130]. The possible role of endocytosis in the control of the ligands has also been investigated, since E3 ubiquitin-ligases Neuralized and Mind Bomb were found to be required for ligand internalization, causing higher activity of the ligands on the cell surface [135-136]. Interestingly, the Notch extracellular domain undergoes trans-endocytosis into the ligand-expressing cells, dissociating from the transmembrane domain which undergoes further processing [137]. Several hypotheses have been proposed to explain the link between ubiquitination, endocytosis and ligand activity: ligand endocytosis could generate a pulling force on the extracellular domain of the Notch receptor, therefore triggering the exposition of the cleavage site for ADAM proteases; ligand ubiquitination could promote its clustering and therefore a more robust Notch activation, or the trafficking in the endocytic compartment could allow post-translational modifications on the ligands improving their activity [138] (schematic representation of Notch pathway in **Fig.8**).

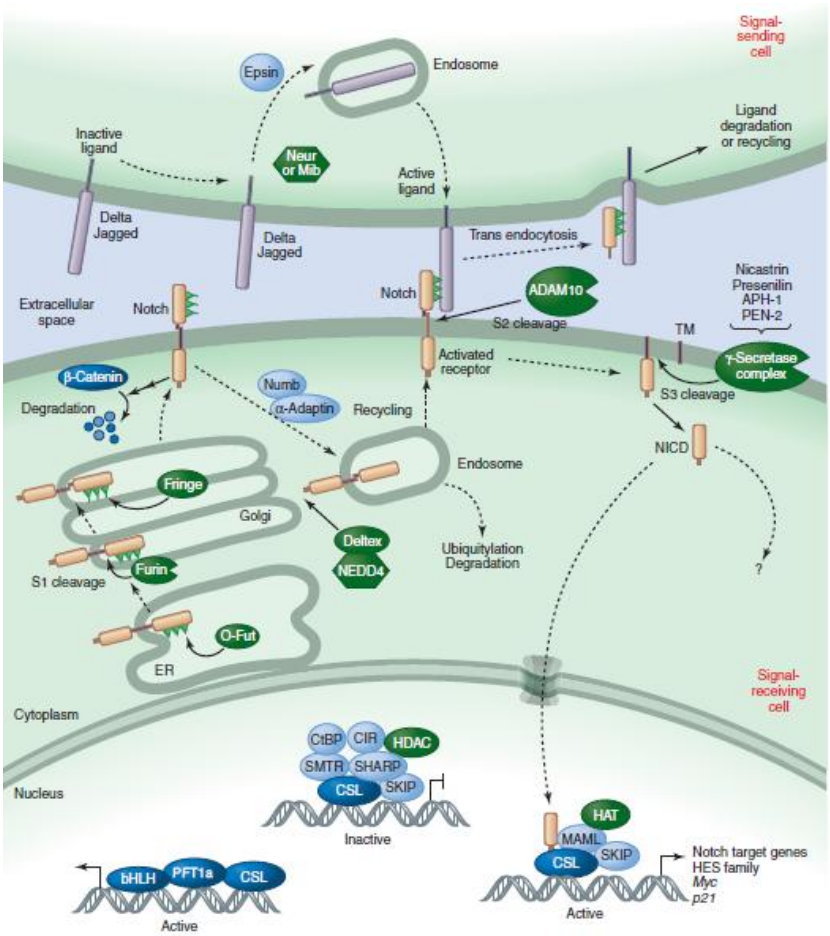


Figure 8. Notch trafficking and signaling pathway (adapted from [139]).

Notch function

Notch signaling is highly evolutionary conserved and plays a crucial role in the embryonic development of flies, worms and mammals. Its signaling pathway regulates many processes of cell fate decision; in the context of binary cell fate decision, Notch regulates lateral inhibition between adjacent cells, which means that a population of cells with equivalent developmental potential will signal to each other through inhibitory reciprocal Notch signaling. By the amplification of differences in the expression levels of either ligand or receptor within the cell population, one of the cells will acquire a higher level of the ligand amplified by

feedback regulatory loops, therefore inhibiting the neighboring cells to adopt the same fate using Notch signaling [140-143] (**Fig. 9A**). Another case of binary cell fate occurs when both sister cells express ligand and receptor but, due to asymmetric segregation of cell fate determinants which can negatively regulate Notch signaling occurred prior to mitosis, the signal is polarized and the cells undergo different fates. One of the most important Notch-inhibitory factors is Numb, a phosphotyrosine binding domain adaptor protein able to bind Notch and to prevent its activation promoting the receptor degradation (**Fig.9B**) [142, 144-145].

Notch signaling is also involved in the boundary specification through inductive signaling. This mechanism of signaling results in the creation of a new cell type after cell-cell interactions at the boundary between distinct cell populations (**Fig.9C**) [142, 146-147].

Notch exerts a pivotal role in the maintenance of stem cell populations. In order to maintain tissue homeostasis, within a population of stem cells, some have to undergo differentiation, while others have to maintain the stemness features. This is achieved by asymmetric cell division mediated by Notch signaling in many embryonic and post-embryonic stem cell compartments (**Fig.9D**) [112, 148-149].

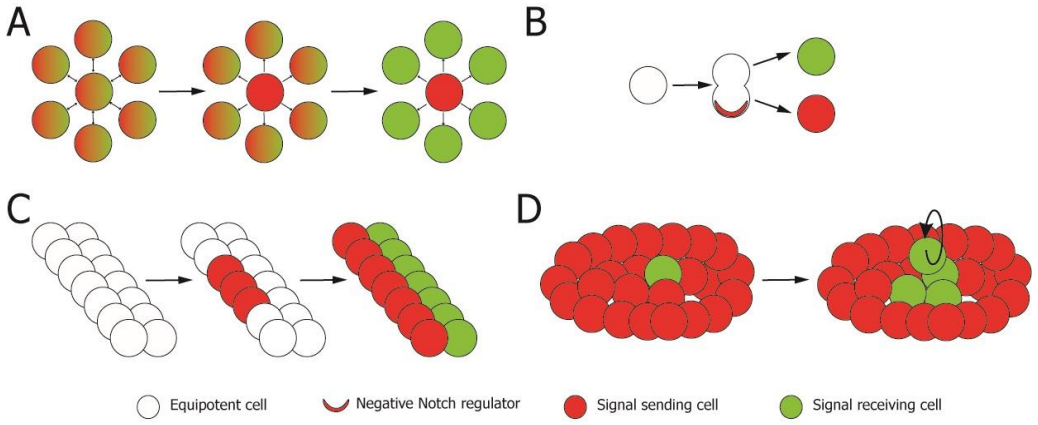


Figure 9. Binary cell fate decision (A and B), inductive signaling (C) and stem cell pool mantainance (D) regulated by Notch signaling pathway.

More in detail, the importance of Notch signaling in the stem cell context has been widely reported in the nervous system, in Drosophila and in mammals. In Drosophila, the Notch signaling is responsible for the fate decision of individual cells among an equipotent cluster in the ectoderm, specified to become neuronal progenitors [150]. During embryonic development in mice, Notch stimulates precursor cell proliferation and inhibits neural differentiation promoting glial cell fate, while later it stimulates differentiation of astrocytes and inhibits terminal differentiation to oligodendrocytes [151]. In adults, it exerts a fundamental role in maintaining the stem cell pool present in the subventricular zone and in the subgranular zone of the brain. Here, it regulates the cell cycle exit of neural stem cells. Blocking Notch results in the exhaustion of the stem cell pool, with an increased differentiation into transient amplifying cells and neurons [152-153]. This mechanism might exert a role in the expansion of the neural stem cell pool after injury [154].

In the intestine, stem cells reside in the Lieberkühn crypts. Here, Notch plays two independent roles. It regulates stem cell proliferation, since its activation triggers the amplification of stem/progenitor cell pool through the action of its target gene Hes1 which blocks the expression of cyclin dependent kinase inhibitors [151, 155]. Moreover, it inhibits the differentiation toward secretory cells and favors the differentiation toward the absorptive phenotype via the negative regulation on the transcription factor Atoh1, which promotes secretory cell fate commitment [156-157].

During muscle development, a pool of progenitors exit from the cell cycle and undergo differentiation expressing specific transcription factors and forming multinucleated myotubes. Satellite cells expressing the Pax7 transcription factor are localized adjacent to muscle fibers under the basal lamina, where they remain quiescent. This compartment is crucial in the regenerative process after injury, since satellite cells are driven back into the cell cycle and induce myotube proliferation. In vitro, activation of Notch signaling leads to the prevention of multinucleated

myofiber formation, therefore suggesting that Notch activation blocks differentiation [158-159]. Loss of Notch signaling is accompanied by the loss of the satellite cell population since the pool of progenitors undergoes exhaustion [160]. Moreover, the satellite cells fail to assume the correct position, pointing to a role of Notch signaling also in the homing of stem cells to the correct niche [161]. In the adult, Notch pathway was demonstrated to be active in quiescent satellite cells, where it probably represses the key transcription factor necessary for terminal differentiation [162-163]. In the case of injury, Notch is down-regulated and the satellite cells exit from the quiescent state to differentiate and contribute to regeneration [164].

Finally, Notch signaling has been demonstrated to play a pivotal role in hematopoietic stem cell compartment in the embryo, where it is essential for generating hematopoietic stem cells (HSCs) from endothelial cells in the aorta-gonad-mesonephros regions [165]. In the adult, the role of Notch is more controversial. Notch signaling has been reported to affect self-renewal, proliferation and differentiation of adult HSCs, since the treatment with its ligand increases HSC expansion in vitro [166-167] and overexpression of active Notch preserves/expands hematopoietic progenitors in vivo [168-169]. Opposite to these results, several genetic studies could not identify any crucial role for Notch signaling in HSC maintenance or proliferation [170-171], supporting the idea that it is not necessary for homeostasis under steady-state conditions, while it is a potent tool to expand HSCs in vitro.

During somitogenesis, Notch also regulates temporal synchronization of the development of a group of cells: expression of the downstream gene Hes1 oscillates and Notch, while not triggering the oscillation itself, controls the synchronization of the oscillating signals [172-173].

The role of Notch during heart embryogenesis and in the adult life

Notch signaling exerts different important roles during cardiac morphogenesis. In the initial phase of development, Notch signaling acts as an inhibitor of

differentiation towards the cardiomyocyte fate. In different models, such as *Xenopus*, chick embryo or mouse embryonic stem cells, the Notch pathway is able to inhibit cardiac differentiation and myocardial gene expression, blocking mesodermal commitment and cardiac differentiation [174-177]. Furthermore, expression of activated Notch in cardiac mesoderm leads to abnormal morphogenesis due to impaired cardiomyocyte differentiation and hyperplasia of the atrioventicular cushions [178]. Since Notch is involved in binary fate decision, prevention of cardiogenic commitment could result in commitment to another lineage. Indeed, embryonic stem cells receiving a positive Notch signal undergo neurectodermal transition and neural specification and become incapable of adopting a cardiogenic fate [179-180]. The exact mechanism by which Notch inhibits cardiogenesis is not completely defined. Notch target Hey transcription factors are able to block cardiac gene transcription either interacting (and thus inhibiting) the cardiac activator Gata4 or its target genes or binding the Gata4 responsive promoters [181-182]. Moreover, Notch can inhibit Mef2C, which marks the cardiac and skeletal muscle lineages during mouse embryogenesis [162], by either physically binding to it thus impeding its transcriptional ability, or competing for members necessary to form the Mef2C transcriptional complex [183]. During cardiac development, different Notch receptors are expressed with a distinct local and temporal pattern: Notch1 and -2 are mostly expressed in the developing heart, Notch3 is restricted to smooth muscle and Notch4 to the endothelium of the vascular system [184-185]. The ligands also have a , in the ventricular trabecolae and in the atrial myocardium, while Delta-like1 and Delta-like4 are expressed respectively in the endocardium and in the cardiac crescent and later in the ventricular endocardium [186]. Notch and its ligand are highly expressed in the non-myogenic dorsolateral domain of the primary heart field, where they act by suppressing cardiogenesis [174]. Loss of function studies have demonstrated that Notch is not necessary in the first phases of heart development, such as heart field specification and induction of cardiac mesoderm, since complete lack of Notch signaling due to RBP-Jk mutation is not lethal at this stage [187]. The

first defect linked to Notch impaired signaling is the random looping of the heart [188]. Defective Notch signaling also results in defective EMT during the formation of cardiac cushions. This process is essential for the correct development of the endodermal cardiac cushions, from where cardiac valves and atrial and ventricular septa will develop. Notch1 mutants show a defective induction of EMT, with very few migrating cells, lacking mesenchymal morphology [189-190]. The effect of Notch signaling in this process is probably due to Notch target genes, which are able to regulate the expression of specific metalloproteases required for cell migration and to the Notch-mediated negative regulation of cadherin expression, enabling the cells to invade the cardiac jelly [189-190]. During ventricular trabeculae formation, Notch1 is expressed in the endocardium and triggers cardiomyocyte proliferation through two different pathways: it stimulates the production of Neuregulin1 by the endocardium, which allows the transition of primitive myocardial epithelium to trabecular and compact myocardium, and it stimulates BMP10 production in cardiomyocytes, which positively regulates their proliferation [191]. The importance of the Notch pathway in cardiac morphogenesis is highlighted by the strong phenotype of the gain- and loss-of function mutants of the several key molecules of the pathway as well as by the features of the Alagille syndrome, a human autosomal disorder characterized by hepatic, cardiac, skeletal and eye malformation due to Jagged1 mutation. In the cardiovascular system, the abnormalities include ventricular septal defects and hypetrophy of the right ventricle due to underlying pulmonary stenosis [192]. In addition, Notch can also control differentiation of committed mesodermal progenitors and of cardiac precursor cells into cardiomyocytes, reinforcing the conclusion that the Notch pathway is able to control cardiogenesis at multiple steps of the differentiation process, from the mesodermal versus neuroectodermal commitment to myocyte differentiation and proliferation [178-179, 193].

In the adult heart, several members of Notch family are expressed at different levels and Notch signaling is detectable in cardiac non-myocytes and rarely in

cardiomyocytes [186]. It is mainly involved in the maintenance of adult heart tissue integrity. Given the role of Notch in preserving the stem cell pool in several tissues, Notch has been hypothesized to play an important role in cell-to-cell contact signaling between accessory cells and cardiac precursors in putative stem cell niches. In particular, it has been proposed that Notch could be involved in keeping the precursor population quiescent, while maintaining the proliferation and expansion of undifferentiated cardiac precursors cells, eventually leading them from the immature phenotype to the compartment of amplifying myocytes [194-195], also regulating their proliferation and expansion [196].

Several studies have analyzed the role of the Notch signaling in vivo, in the context of cardiac regeneration, reporting a variety of pro-regenerative roles. In a model of myocardial injury, Notch signaling was reported to promote cardiomyocyte survival [197] and contribute to protective signaling, interacting with the c-Met/Akt pathway [198]. It was also reported to inhibit excessive fibrosis in a model of myocardial infarction [197] and of pressure overload, through activation of Notch signaling in heart stromal cells [199]. In a model of aortic constriction, it could play a role in the mobilization of epicardial cells, which contribute to the resolution of fibrosis; in addition, these cells also display a modest differentiation potential towards cardiomyocytes [200].

Finally, Notch has also been proposed to play a role in the mobilization of different population of stem cells, including bone marrow-derived stem cells in the case of myocardial infarction [201], and cardiac precursor cells [194, 199, 202]**.**

Several reports have assessed the role of Notch in the tuning of cardiomyocyte proliferation. Reports from our laboratory have shown that Notch1 drives proliferation and expansion of neonatal rat cardiomyocytes [196]. Moreover Notch ICD was reported to activate Cyclin D1 transcription and also to promote its nuclear localization, therefore stimulating cardiomyocyte cell cycle progression [203], although recent evidence has shown incomplete cardiomyocyte proliferation [197]. In differentiated cardiomyocytes, Notch inhibits hypertrophy, probably by impeding,

through its target gene Hey2, the pro-hypetrophic activity of Gata4. Therefore, Notch might maintain cardiac tissue homeostasis by limiting the extent of the cardiac hypertrophic response [181-182, 204].

EPIGENETIC CONTROL OF GENE EXPRESSION

In eukaryotic cells, nuclear DNA is wrapped around proteins to form the chromatin. The primary protein components of chromatin are histones, assembled in octamers to form nucleosomes. This structure ensures chromatin compaction and it can undergo many modifications regulating most of DNA-related processes, such as transcription, recombination, DNA repair, replication. The modifications can be covalent modifications of the histones, modulation of DNA accessibility through chromatin complexes called chromatin remodelers, or modification on the DNA itself by the addition of methyl groups on the cytosines in the context of CpG dinucleotides. Given the complexity of the topic, in this introduction I will mainly describe the chromatin modifications impacting on transcription.

Histone modifications

Chromatin structure is highly complex and impressively dynamic. The nucleosome and its histone core, which were once thought to be static and highly stabilized by the 14 non-covalent bonds occurring between DNA and histones, actually play an integral role in directing some elements of transcriptional specification. The histone octamers forming nucleosomes are characterized by 15-30 amino-terminal residues which protrude from the nucleosome to form the so-called "histone tails" [205]. More than 60 different residues mainly residing in the histone tails have been demonstrated to be modified by covalent binding of various functional groups.

The most common additions are acetylation, methylation, phosphorylation and ubiquitination of residues, which can act combinatorially, according to the so-called "histone code" [206]. The best characterized mechanisms through which histone

modifications influence chromatin are the disruption of the contacts between nucleosomes and DNA, since many modifications result in the change of the net charge of the nucleosome and in the loosening of the coil, and the recruitment of non-histone proteins, which can combinatorially bind to the modified histones (reviewed in [207]).

Histone acetylation

Histone acetylation is a highly dynamic process known since the 1960s [208]. The class of enzymes responsible for histone acetylation are the Histone Acetyl Transferases (HATs): they utilize AcetylCoA as a cofactor to transfer an acetyl group to the ε-amino group of lysine, therefore neutralizing its positive charge. HATs are divided into 2 families, type-A and type-B HATs. Type-B HATs are mainly cytoplasmic and are able to acetylate newly synthesized histones (K4 on histone 3 and K12 on histone 4), prior to their assembly to form the nucleosome. Type-A HATs are divided into 3 subfamilies according to their sequence homology: GNAT (Gcn5-related N-acetyltransferase), MYST (MOZ, Ybf2/Sas3, Sas2, and Tip60) and CBP/p300; they have a limited substrate specificity, since they are able to add acetyl groups to many different lysine residues. The modified residues are mainly in the N-tail of the histones, and the neutralization of the positive charges given by the acetylation destabilizes the interaction between the nucelosomal proteins and the DNA [207]. There are also additional sites for acetylation within the globular structure of histones, facing the DNA major groove, such as K56 on histone 3 acetylated by Gcn5 in humans, which results in the disruption of the electrostatic interactions between DNA and histone cores, starting DNA unwrapping [209-210].
Acetylation of both histones 3 and 4 has been identified as a mark for active chromatin and HATs activity has been characterized in this context. Gcn5 is a part of an activator complex called SAGA (Spt-Ada-Gcn5 acetyltransferase) and is able to acetylate H3 and H2B, while Esa1, part of nucleosome acetyltransferase H4 complex

(NuA4), preferentially acetylates H4 and H2A. Both complexes are recruited by specific activators to the promoters of genes that are transcriptionally active [211].

The cooperation between HATs and chromatin remodeling complexes has been described in several cases, since acetylated histones are known to be binding sites for other proteins involved in gene regulation, such as the SWI/SNF remodeling complex, which is able to bind and acetylate histones and is involved in nucleosomal mobilization [212]. Tip60 has been identified together with Brg1 (a SWI/SNF chromatin remodeler family member) as a factor affecting the regulation of several developmental genes [213]. Moreover, the presence of other histone modifications such as H3K4 methylation (in the promoter) and H3K36 methylation (in the coding region) can also play a role in HAT recruitment, since proteins able to recognize methylated residues also take part in the formation of HAT-containing complexes [214].

The enzymes responsible for the reverse reaction, namely the removal of the acetyl groups, are named Histone Deacetylases (HDACs), and their action restores the positive charge of the lysine, therefore stabilizing the chromatin structure. HDACs are divided into four classes: class I, II and IV share some sequence homology and need Zinc as a cofactor; class I and II are homologous to yeast (sc)Rpd3 and scHda1, class IV is composed only by HDAC11, class III (also called Sirtuins) are homologous to scSir2 and are NAD+ dependent (extensively reviewed in [215-216]). HDACs not only act on chromatin, but are involved in the tuning of multiple intracellular signaling pathways, showing low substrate specificity. At the transcriptional level, HDACs are able to induce repression of transcription not only by restoring the net positive charge of the lysine residues, therefore reducing the affinity of histones for several transcription factors, but also increasing the affinity for transcriptional repressors, such as proteins containing the SANT domain, which recognizes unmodified histones [207, 214, 217].

To conclude, paradoxically, HATs are also found at inactive gene promoters, where they probably prime the genes for activation [218] and HDACs are also important in

active genes, since they are required to ensure proper transcription initiation and to commit genes to rapid repression [214, 219]. The pattern of histone tail acetylation can also regulate chromatin compaction dynamics, with a direct effect on chromatin structure, since there are evidences that acetylation in H4K16 inhibits the formation of the compact 30 nm fibers [220-221].

Histone phosphorylation

Histone phosphorylation is a highly dynamic process occurring on serine, threonine and tyrosine residues present in the N-tail of histones, able to modify the net charge of the chromatin thanks to the transfer of a negatively charged phosphate group on the side chain of the target amino acid. Several cellular kinases have been found to bind chromatin, suggesting their active role in chromatin phosphorylation [222]. Phosphorylated residues in the histone tails behave as docking sites for signaling effectors and adaptors, as 14-3-3 proteins, which have been characterized as downstream effectors [223]. Histone phosphorylation plays a role in different processes, including gene expression, DNA damage response and chromatin compaction (reviewed in [224]).

Regarding the regulation of gene expression, H3S10, T11 and S28 phosphorylation is associated to Gcn5-dependent histone acetylation, strongly supporting the existence of a crosstalk between acetylation and phosphorylation to promote transcription activation [224]. In some cases, activation of the transcription can be achieved through an allosteric regulation of the protein complexes present on the chromatin. As an example, H3S28 phosphorylation can be correlated to transcription activation through the displacement of the Polycomb repressive protein complex, which acts on the upstream amino acid (H3K27) in the histone tail. Moreover, H3Y41 phosphorylation activates transcription by disrupting chromatin binding by the repressive protein HP1 [224].

Histone ubiquitination

Ubiquitination, most frequently monoubiquitination, is a covalent modification occurring on lysine residues of the histone proteins assembled as octamers in the nucleosomes, as well as in the linker histone H1. The best characterized is the role of H2AK119 and H2BK120 (K123 in yeast) ubiquitination [207]. H2A-ubiquitin ligases are often found in transcriptional repressor complexes, as it is the case for Ring1, a member of gene silencing-related Polycomb Group protein 1, discussed later in details [225], while H2B ubiquitination is associated to active gene expression, promoting transcriptional activation and elongation [226-227].

Histone methylation

Histone methylation consists in the transfer of a methyl group from S-adenosylmethionine to the side chains of lysine or arginine residues; this modification does not affect the net charge of chromatin, but creates docking sites for different combinations of interacting proteins, able to modulate the downstream signaling. This chemical modification shows an additional level of complexity, since lysine residues can be mono-, di- and trimethylated, while arginines can be mono-, symmetrically or asymmetrically dimethylated [207]. This allows a wide combination of interactors to be recruited and be responsible for modulating the transcriptional cascade.

Arginine methylation

There are two classes of arginine methyltransferase (PRMTs): type-I PRMTs are responsible for monomethylation and asymmetric dimethylation, while type-II PRMTs are responsible for monomethylation and symmetric dimethylation [228]. PRMTs are recruited to promoters through specific transcription factors [229] and are able to methylate not only histones but transcription factors and coactivators as well, therefore impacting on a wide range of effector molecules. The role of arginine methylation can be linked to either active or repressed transcription. In the case of

H3R2 methylation, transcription is inhibited, since the K4 methyltransferase complex Mll1 does not methylate H3K4, a known mark of active chromatin [230]. To date, few interacting proteins with methylarginine residues have been described. Recent evidence suggests that the de novo DNA methyltransferase Dnmt3A can recognize symmetrically methylated H4R3, therefore promoting DNA methylation and gene silencing [231]. A potential binding protein for asymmetrical arginine methylated histones has been recently identified in Tdrd3, which is able to recognize methyl marks associated with transcriptional activation (H3R17me2a and H4R3me2a), possibly being recruited on active gene promoters [232].

Lysine methylation

Many different enzymes responsible for lysine methylation have been identified, called histone lysine methyltransferases (HKMTs), divided into two families: the Set-domain-containing protein family and the non-Set-domain-containing family Dot1 [233]. Their target sites mainly reside in the N-tail of the histones, with the exception of H3K79 which is inside the globular structure [207]. HKMTs display a specific substrate specificity, being able to methylate only one specific residue; moreover, they can be so specific to catalyze only one state of the methylation (for example, Set7/9 is only able to monomethylate its target H3K4 [234]).

Methylation has been considered for long time as a static modification, but since 2002 many demethylases have been identified, for both lysine and arginine. The first identified lysine demethylase is Lsd1 (Lysine-specific demethlyase 1), which is only able to demethylate mono- and di-methylated substrates, having different functions according to its molecular partners. It can demethylate both H3K4 me1/2 and H3K9, so it can act both as a repressor, in complex with Co-REST, and as an activator of transcription [235-237]. In 2006, another class of enzymes, able to demethylate trimethylated lysines, was discovered [238-239]. The first identified member was Jumonji domain 2 (Jmjd2), able to demethylate H3K9me3 and H3K36me3. Its enzymatic activity resides in the JmjC domain, which is shared by all the known

lysine demethylases except Lsd1. All these enzymes also display a high level of substrate specificity [240].

Lysine methylation has been linked to either active or inactive transcription. Methylation of H3K36, H3K79 and H3K4 are correlated to transcriptional activation, H3K4 and H3K36 methylation are also involved in transcriptional elongation.

H3K36 methylation, which can be mono-, di- or trimethylation, has also a role in preventing transcription from inappropriate sites, since it has been reported that Eaf3 (subunit of Rpd3S histone deacetylase complex) binds di- and trimethylated H3K36 (H3K36me2 and me3), therefore moving the HDAC complex to the coding region [219, 241-242].

H3K79 methylation is catalyzed by Dot1 and can be present as H3K79me1, -me2 or -me3. Interestingly, H3K79 and H2BK123, a residue which can undergo ubiquitination, lie in the proximity of the same exposed nucleosome surface; an interplay of the two modifications has been demonstrated, since H2BK123Ub is a prerequisite for H3K79 and H3K4 methylation [243]. Its genome-wide localization analysis reveals an enrichment in gene coding regions, consistent with its deposition concomitant to the elongation by RNA Pol II.

H3K4 is methylated mainly by Set1 in the ORF of active genes [220]; monomethylated residues are predominant at the 3' of the ORF, dimethylation peaks in the middle and trimethylation surrounds transcription starting sites and the 5' of the ORF, therefore positively correlating with gene expression [244]. The methyl mark at the 5' of the ORF seems to be critical for transcription initiation and Pol II recruitment. H3K4 does not influence elongation or Pol II activity per se, but H3K4 methylated histones are recognized by chromatin remodeling complexes as NURF [245] and this modification prevents the association of negatively acting nucleosome remodelers and histone deacetylation complexes (NuRD) [246]. Methylated H3K4 is also recognized by Isw1, a subunit of the Isw1 ATP-dependent chromatin remodeling complex [247] and by members of SAGA complex (an acetyltransferase complex) involved in transcriptional activation [248-249]. Interestingly, the presence

of methylated H3K9 impairs the methylation of H3K4, possibly making electrostatic interaction with the HKMTs, therefore preventing its action on H3K4 [250].

Lysine methylation marks associated with transcriptional repressions are H3K9, K3K27 and H4K20 [233].

H3K9 is linked to heterochromatin subdomains and gene silencing. Transcriptionally inert regions at pericentric chromatin are marked by H3K9 methylation, which is recognized by Heterochromatin Protein 1 (Hp1) [251-252]. It is also linked to X chromosome inactivation [253-254]. An interesting link between H3K9 methylation and DNA methylation has been proven in a variety of organisms: in *Neurospora Crassa,* HKMT Dim-5, which methylates H3K9, is necessary to direct DNA methylation [255-256]. In mammals, at peri-centromeric chromatin, DNA methylating enzymes are recruited to H3K9-methylated heterochromatin through direct interaction with Hp1 [257]. H3K9 methylation is not restricted to constitutive heterochromatin, since HKMT are active also in repressing euchromatin targets, through the recruitment of HP1 at the promoter of repressed genes [258]. H3K9 methylation can be present as mono-, di- or trimethylation (H3K9me1, me2 or me3 respectively). The methylation distribution has been extensively studied, demonstrating that H3K9me1 is enriched in gene promoters and 5' UTR regions, while it is minimal in intergenic regions; H3K9me2 is a mark of heterochromatin, prevails at peri-centromeric and subtelomeric regions, but also covers large genomic areas encompassing coding and non coding regions; H3K9me3 has previously been found in heterochromatin regions and on repressed promoters, but more recently has been shown to be present also at gene bodies of actively transcribed genes, together with Y-isoform of Hp1 [259-260].

H4K20 is the only methylation site on histone 4. Depending from the degree of methylation, H4K20 can exert different roles. K20me1 has been linked with transcriptional repression [261], cell cycle regulation [261-262], X chromosome inactivation [263] and nucleosomal compaction. H4K20me2 plays a role in the cellular response to DNA damage [264], acting as binding site for proteins needed in

DNA double strand breaks response. H4K20me3 is highly enriched in pericentromeric heterochromatin, in combination with H3K9, probably acting as binding sites for proteins involved in heterochromatin formation; interestingly the HKMTs responsible for H4K20 methylation are not able to act on pericentromeric chromatin in the absence of HP1 and H3K9 methylation [264-265].

Polycomb Group (PcG) proteins

H3K27 can be found in mono-, di- or trimethylation state, and the distribution of the different methylation levels is not homogenous: H3K27me1 is enriched at pericentromeric chromatin, at major and minor satellite repetitive elements as H3K9me2 and H4K20me1 and in X-inactivated chromosomes; H3K27me2 and H3K27me3 are spread in silent regions of euchromatin; moreover, H3K27me3 is absent in repetitive elements while it regulates directly many key genes in development and differentiation. H3K27 methylation was discovered as a regulator of the homeotic genes in Drosophila, where repression of the developmentally regulated *Hox* genes by the proteins of the Polycomb Group (PcG) was discovered as antagonists of Tritorax-mediated transcriptional activation [266]. It became later clear that a wide variety of genes are associated to this modification. The core complex responsible for H3K27 methylation is Polycomb Group of proteins, well conserved from Drosophila to mammals [267-271]. PcG proteins are mainly assembled into two multicomponent complexes, called Polycomb Repressive Complex 1 (PRC1) and Polycomb Repressive Complex 2 (PRC2). PRC2 is composed of Suz12, Eed, RbAp46/48 and Ezh1/2, which is the enzyme responsible for methylation of H3K27 (**Fig.10, left**). Ezh2 is essential to di- and tri-methylate H3K27, while it is dispensable for monomethylation, possibly carried out by a complex involving Ezh1 [272-274]. Some additional proteins are associated with PRC2: Aebp2 enhances the enzymatic activity of the complex, PCL is able to interact with PRC2 through Ezh2, and Jarid2, a member of the Jumonji family of lysine demethylases, lacks the residues necessary for cofactor binding, therefore is devoid

of catalytic activity. This protein interacts with Ezh2, and probably has an inhibitory effect on lysine methylation mediated by PRC2; it recognizes similar sequences to the PRC2 target sequences, therefore it can have a role in the recruitment of PRC2, as well. PRC1 in mammals is composed by the Cbx (Chromobox-domain) protein, which recognizes H3K27me3 through a chromodomain, by one member of PCGF family, of the Ring family (Ring1a and -1b) and of the Hph family. Ring proteins are responsible for the ubiquitination of lysine 119 of H2A (**Fig.10, right**).

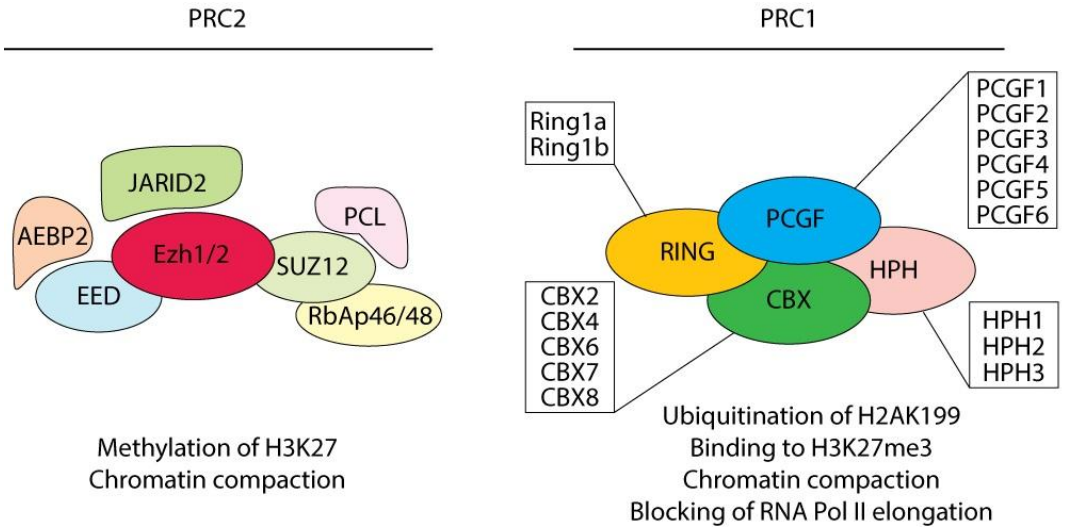


Figure 10. Components of PCR2 and -1.

The common model of recruitment of PcG complex implies that its target genes are recognized by PRC2, which methylates H3K27. This mark acts as a binding site for PRC1 through Cbx; the PRC1 member Ring has a ubiquitin E3 ligase activity specific for lysine 119 of histone 2A. This triggers an inhibition of the transcription either by recruiting silencing proteins or by interfering with initiation or elongation steps [225, 275-276].

Interestingly, even if the Polycomb mediated silencing mechanism is well conserved through evolution, the mechanism of recruitment of PRC to its target is not. In Drosophila, PcG protein complexes are recruited by DNA motifs called Polycomb response elements (PRE), recognized by a variety of transcription factors, mainly not conserved in mammals. PRC2 seems to target CG rich regions, showing an almost

perfect correlation with the CG-rich elements [277]. Several mechanisms have been proposed to explain PRC2-targeting to these regions: the core components of PRC2 complex could act synergistically to target it to CG-rich regions, since they show moderate affinity of CG-rich stretches; sequence specific transcription factors could regulate PRC2 binding to the targets; long non coding RNAs could play a role in PcG proteins recruitment, since it has been reported that the long non coding RNA Xist can interact with PRC2 [176]. Recent evidence has however reported the identification in mammals of sequences recognized by PcG showing similarity with the Dorspohila PRE, suggesting an important role for Yy1, the mammalian orthologue of Drosophila PRE DNA-binding protein Pho [278-279]. It was recently shown that non-canonical PRC1 complexes are not only dependent on H3K27 methylation for their recruitment, but can also interact with sequence specific DNA-binding proteins, which could target the complex to a specific site on the genome [280].

H3K27 methylation has been implicated in various biological processes, such as X chromosome inactivation, genomic imprinting and silencing of developmentally regulated genes [233]. More in detail, genome-wide mapping of PcG complexes shows that they are bound to the majority of the genes involved in key developmental pathways and reside at the promoters of a very large number of tissue-specific differentiation genes [281] playing a key context-dependent role in the maintenance of stem cell proliferation and in many differentiation processes [282-283]. Interestingly, H3K27 trimethylation correlates with PRC2 promoter occupancy and covers up to 20% of embryonic stem cell promoters. These promoters, however, are also marked by H3K4me3, correlating with active transcription [284-285]. These are the so-called chromatin "bivalent domains", well described in embryonic stem cells. They cover genome regions where genes are "primed" to be quickly activated upon arrival of differentiation signals, but in the absence of a differentiation cue, they are ready to be repressed. After cell fate

decision, bivalent "genes" lose the active marks and repress transcription through H3K27me3.

The PRC2 complex can contain alternatively the Ezh1 or Ezh2 methylating enzyme. PRC2-Ezh1 and PRC2-Ezh2 occupy an overlapping set of genes, probably co-occupying them, but their expression profile is very different; Ezh1 is ubiquitously expressed, while Ezh2 is strongly associated with proliferative tissues. Ezh1, which is able to efficiently methylate H3K27 in vitro, shows very low levels of methyl-transferase activity in vivo. Indeed, Ezh2 knockdown strongly affect global H3K27me2/me3 levels. PRC2-Ezh1 acts by strongly repressing transcription through the induction of chromatin compaction. It has been proposed that the PRC2-Ezh2 complex establishes the de novo methylation pattern, being later downregulated, with PCR2-Ezh1 and PRC1 keeping the chromatin repressed [201, 273].

A recent report hypothesized a completely new function for Ezh1. The authors characterized Ezh1 occupancy genome-wide in myoblasts and in myotubes, and noticed that the majority of Ezh1 sites overlapped with H3K4me3 and RNA Pol II sites, suggesting that genes bound by Ezh1 are actively transcribed [286]. This observation reveals a potential role for PcG complexes in promoting mRNA transcription, in contrast to the well-documented role of PcG as transcriptional repressors.

ATP-dependent chromatin remodeling complexes

The ATP-dependent chromatin remodeling complexes use energy derived from ATP hydrolysis to move, destabilize, eject or restructure the nucleosomes. The need for chromatin remodelers is due to the necessity to have a regulated DNA accessibility in many different contexts, such as after replication, when the nucleosomes have to be placed at the proper distance; during DNA repair, when nucleosomes have to be ejected or shifted to provide access to chromatin; in the exposition of normally unexposed sequences which have to be bound by a variety of factors; during the progress of DNA or RNA polymerase, when the presence of nucleosome could

impede their progress and they have to be displaced and later repositioned in the proper place.

The chromatin remodelers share a conserved ATPase subunit homologous to the ATPase domain of ATP-binding helicases of the DEAD/-H family, comprised of two parts separated by a linker [287]. According to the additional presence of a unique domain adjacent to the ATPase domain, involved in recognition of modified histones, modulation of ATPase activity and interaction with chromatin, chromatin modifying enzymes and transcription factors [287-288] they are then classified into 4 different families called ISWI (imitation switch), CHD (chromodomain, helicase, DNA binding), INO80 (inositol requiring 80) and SWI/SNF (switching defective/sucrose nonfermenting) (**Fig.11**).

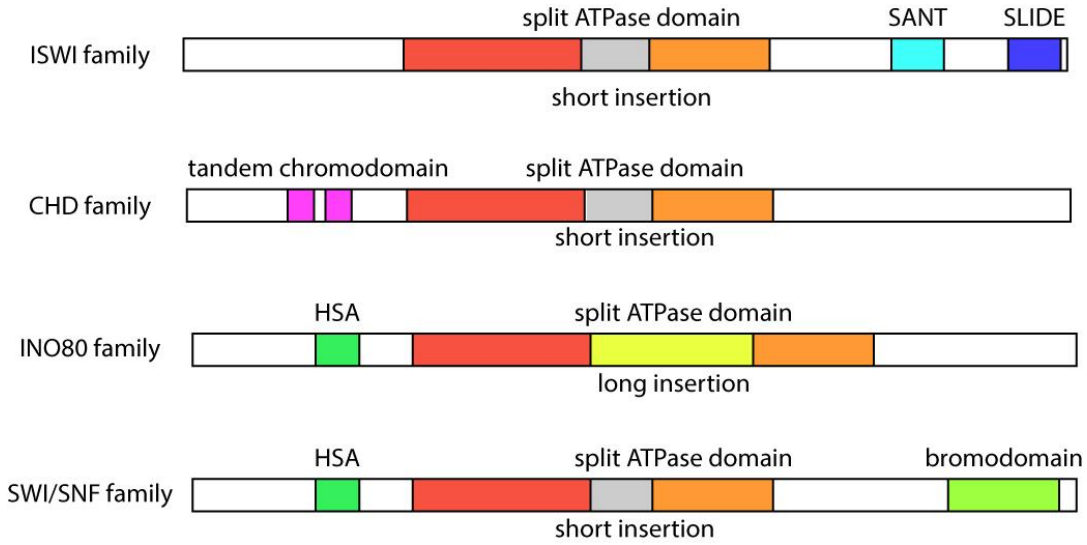


Figure 11. Chromatin remodeler families, defined by their unique domains adjacent to the ATPase domain.

ISWI remodelers are composed of 2 or 4 units. Beside the conserved ATPase domain, ISWI complexes share the presence of a SANT domain adjacent to a SLIDE domain at the C-terminus, acting together to recognize the nucleosome unit, identifying both DNA and histones. ISWI complexes have been firstly described as involved in transcriptional activation: mutants for the ISWI-member NURF complex

exhibit developmental defects related to reduced expression of homeotic genes and NURF has been demonstrated to directly interact with transcription factors [289]. Beside this role of positive regulation of gene expression when ISWI is present in euchromatin regions, they have also been shown enriched in heterochromatin regions, playing a role in heterochromatin formation: mutant ISWI results in decondensed chromosomes in flies, reflecting a role in chromatin compaction [290]. This phenotype is consistent with the role of ISWI to catalyze the deposition of properly spaced nucleosomes. Interestingly, an inhibitory role on genes transcribed by RNA Pol I and III has been demonstrated, and the repression is related to ISWI-mediated recruitment of HDAC1 and DNA methyltransferases [291]. The distinct subunits which can form ISWI complexes possibly explain the opposite role of the processes in which ISWI family is involved [292].

The CHD family is characterized by N-terminal tandem chromodomains in addition to the conserved DEAD/H-related ATPase domain. CHD members are then subdivided into groups, according to similarities in domain structure. Chd1/2 have an additional C-terminal DNA domain; they can act as monomers or dimers, and they interact with HAT related complexes as SAGA and SILK [293-294]. They are targeted to sites of active transcription and associate with pre-initiation factors to favor transcriptional elongation [295]. Consistently, genome-wide binding of Chd1 correlates with H3K4me3 and RNA Pol II in ES cells, where probably is required for the maintenance of an open chromatin state [296]. Chd3/4 lack the DNA binding motif but they share N-terminal paired PHD finger domains; they are incorporated in a large protein complex with histone deacetylases called NuRD. The role of CHD is to facilitate the access to acetylated histones on the chromatin for the action of HDACs [297]. Chd5 is predominantly expressed in neural tissues and in testis and has been characterized as a tumor suppressor associated with neuroblastoma. It probably works by forming a nucleosome remodeling and deacetylation (NuRD) complex that regulates transcription of specific genes [298]. Chd7 seems to be involved in the transcriptional regulation of many key developmental genes and it has been shown

to interact with Brg1 (a SWI/SNF family member) to regulate the neural crest transcriptional program [299-300].

INO80 remodelers were firstly isolated in yeast, in which ino80 mutants are defective for the transcriptional activation in response to inositol depletion [301]. INO80 complexes are composed of 10 subunits [301]. The common core is the split ATPase domain with a long insertion present in the middle of the ATPase domain, which acts as a scaffold for the association of two proteins called Rvb1 and Rvb2, which form a hexameric structure and exhibit helicase activity, binding replication forks [292, 302]. INO80 members also share a N-terminal helicase-SANT-associated (HSA), which acts as a docking site for various interactors. INO80 complexes can slide nucleosome and evict histones from DNA. Srw1 (in yeast, Srcap in human), a member of INO80 family, has the unique ability to restructure the nucleosome by removing canonical H2A-H2B dimers and replacing them with H2A.Z-H2B dimers. They exhibit several functions, as INO80 members can act at the promoter level regulating gene expression both positively and negatively.

The SWI/SNF family has been originally isolated in yeast, where the complex is composed of 8 to 14 subunits. The SWI/SNF complex contains the ATPase Swi2/Sbf2p and other subunits involved in DNA and protein-protein interactions. It is able to alter nucleosome structure in an ATP-dependent manner. The catalytic ATPase included an HSA (helicase-SANT) domain and a C-terminal bromodomain. There is also a closely related complex named RSC (Remodeling the structure of chromatin), which has an ATPase related to Swi2/Snf2p and the protein complex is formed by analogs of the members of SWI/SNF. Interestingly, the RSC and SWI/SNF are found at different chromatin regions, displaying non redundant function [292]. In mammals, there are two homologues complexes to SWI/SNF and RSC called BAF (Brahma-associated factor) and PBAF. The BAF complex has been shown to be fundamental for correct heart development. The complex can include alternatively Brg-1 or Brahma ATPase and is important in the regulation of a variety of morphological processes: in the endocardium it regulates the correct trabeculation

[303], it is also involved in myocardial proliferation in the embryo and in the control of gene expression of embryonic specific genes concomitant with the repression of adult specific genes [304]. Its deletion in secondary heart field precursors results in a hypoplastic right ventricle and in impaired outflow tract [304]; moreover, BAF member Baf60c is essential for the correct looping of the heart [305-306].

DNA methylation

DNA methylation represents another epigenetic modification able to profoundly affect gene expression. In vertebrates, it occurs on position 5 of the cytosine within the dinucletide CpG, leading to the formation of 5-methylcytidine (5mC) (**Fig.12**).

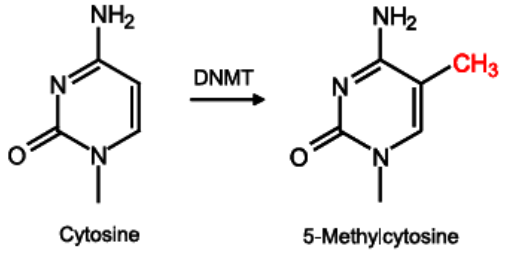


Figure 12. DNMTs catalyze the covalent addition of a methyl group to position 5 of cytosine [307].

Historically, DNA methylation was considered as a defense system of the host genome against retrotransposons. It has also been connected to heterochromatin formation, transcriptional silencing, control of gene expression during development, imprinted gene expression, X chromosome inactivation and silencing of repetitive elements [308].

The discovery of methylation on cytosine residues dates back to 1975, when it was hypothesized that the methylation pattern of DNA can be inherited through somatic cell divisions and that it is related to gene silencing [309]. DNA methylation occurs through the action of a family of enzymes called DNA methyltransferases (DNMTs), which are required and essential for embryonic and neonatal development [310-311]. These enzymes are grouped into two classes, according to their preferred

substrates: Dnmt1 is the maintenance methylase, which recognizes the methylated CpG residues of the hemimethylated DNA generated during DNA replication and methylates the opposite strand, while Dnmt3a and -3b are responsible for de novo methylation, introducing methyl groups on previously unmethylated CpG sites [311-312] (**Fig. 13**).

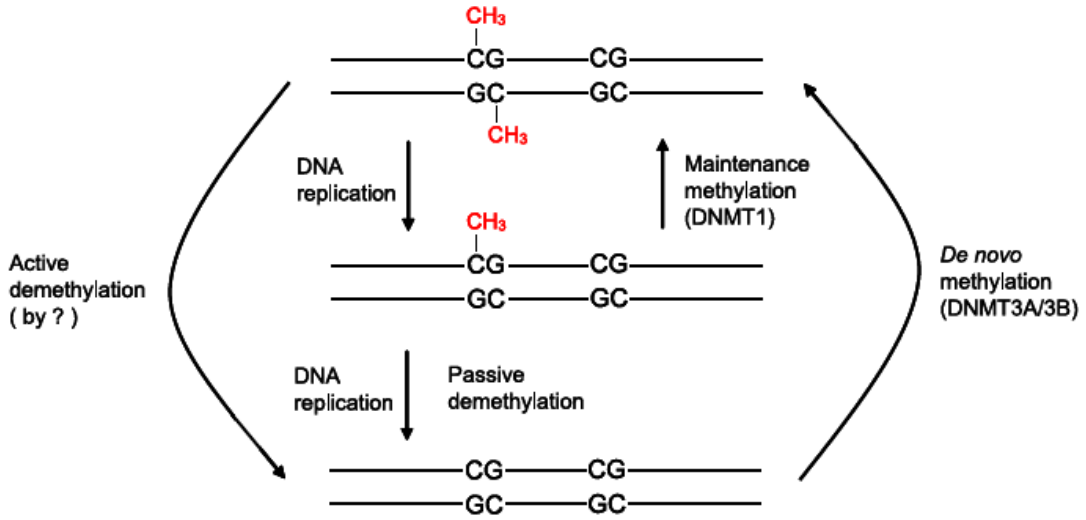


Figure 13. Schematic representation of mechanisms involved in DNA methylation and demethylation [307].

De novo methylation occurs in germ cells and early embryos as a response to the massive wave of demethylation previously occurred. After fertilization, the paternal genome is actively demethylated, while the maternal genome seems to be passively demethylated; after implantation, de novo methylation reestablishes the DNA methylation pattern which will be maintained, in large part, in all the somatic tissues [313-314]. Moreover, de novo methylation occurs during lineage-specific differentiation, such as in hematopoietic progenitors [308, 315]. The same process also regulates genomic imprinting and X-chromosome inactivation.

An essential role in methylation establishment is also attributed to Dnmt3L, a protein that lacks intrinsic catalytic activity but shares sequence similarity with DNMTs. Structural analysis showed that two copies of Dnmt3a form a tetrameric complex

with two copies of Dnmt3L and that this tetramer is able to methylate two CpG sites preferentially separated by 8-10 bps. CpG periodicity is not enough to fully explain how de novo methylation is targeted to specific sequences [307, 316-317]. Targeting of DNMTs can be achieved via multiple pathways. In cancer cells, different factors, as the fusion protein Pml-Rar [318] or c-Myc [319] have been demonstrated to recruit DNMTs, stimulating the methylation of target gene promoters, therefore leading to the hypothesis that DNMTs targeting could be regulated by interaction with repression proteins.

Another mechanism to target de novo methylation is the interaction between DNMTs and histone tails. The repressive mark H3K9me3 is recognized by Hp1, able to recruit Dnmt3A, which catalyzes the methylation of target promoters in embryonic stem cells [320] as well as DNA satellite repeats at pericentromeric heterochromatin [257]. The lack of modification at H3K4 (H3K4me0) is also strongly correlated with DNA methylation: H3K4 methylation prevents de novo methylation, since DNMT3-associated protein Dnmt3L can specifically interact with unmethylated H3K4, and is blocked if H3K4 is methylated [321-323]. Notably, H3K4me3 can act as a binding site for H3K9me2 demethylases; since it seems that Hp1-mediated interaction with H3K9 recruits Dnmt3a/b, this could suggest that an interplay between different histone lysine methylation pattern could influence DNA methylation [324]. The relationship between Polycomb-mediated transcriptional repression and DNA methylation has not been completely elucidated yet. In many cases, DNA methylation and Polycomb silencing seem to be alternative mechanisms, since H3K27 methylation marks in embryonic stem cells coincide with CpG islands, which were thought to be devoid of DNA methylation. However, some evidences show that the two epigenetic modifications can act in cooperation, as in a model of differentiation from embryonic stem cells toward neurons, in which a subset of the genes which are marked by H3K27 trimethylation at the ES-cell state are four times more likely to acquire DNA methylation [325], and in cancer stem cells, in which the promoters marked by H3K27 trimethylation are frequently hypermethylated at the

DNA level [326]. In vitro biochemical data also proved that Ezh2 can bind DNMTs [327], even if it seems that the interaction is not enough to trigger DNA methylation, probably due to the absence of additional regulatory factors of histone marks required for DNMT activity [328]. A recent report suggests that Dnmt3L can interact with PRC2, in competition with Dnmt3a and Dnmt3b, therefore maintaining a hypomethylated pattern at H3K27me3 regions [329].

DNA methylation covers up to 70-80% of the genome. Its pattern is not a continuum along the genome, but displays a bimodal distribution: genomic regions can be hypo- or hypermethylated. CpG methylation can occur in genes, intergenic regions, transposons [330]. The only regions usually devoid of methylation are the so-called CGI (CpG islands), discussed later.

The mechanisms regulating the relationship between silencing and DNA methylation are still not completely clarified. A first hypothesis postulated that the presence of DNA methylation on the cognate DNA sequences can directly inhibit the binding of transcriptional activators [331] (**Fig.14A**). Another hypothesis is that methyl-binding-proteins (MBP) recognize methylated DNA and recruit corepressors to silence gene expression (**Fig.14B**). A third option is that DNMTs, in addition to their enzymatic role in the establishment of the 5mC pattern, couple methylation with the recruitment of enzymes able to modify chromatin, since it has been proven that they can interact with histone deacetylases and chromatin remodeling factors [312, 332] (**Fig.14C**).

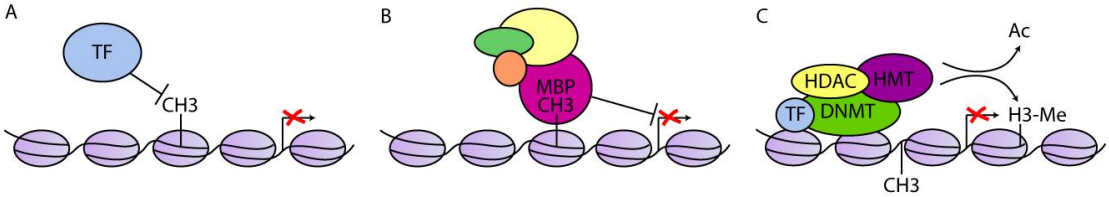


Figure 14. Possible mechanisms linking DNA methylation to transcriptional silencing.

Beside the well established role in silencing, it is now evident that CpG methylation in gene bodies is related to transcription. It has been also proposed that H3K36m3, which associates with transcriptional elongation but not initiation, might be involved in recruitment of DNMTs [333]. Recent work involving whole genome sequencing identified an enrichment of DNA methylation in exons compared to introns, possibly suggesting a role for methylation also in splicing regulation [334].

The best characterized role of DNA methylation involves the methylation occurring at the promoter level. In the mammalian genome, between 55% and 70% of gene promoters are associated with CpG islands (CGIs), which are sequences of DNA with CG content over 55%, approximately 1000 bps long and the often encompassing the transcription start site [330]. CGI promoters are usually unmethylated and transcriptionally permissive. They were originally considered to be a feature of housekeeping genes, therefore never methylated, but it is now evident that tissue-specific genes also have CGI promoters. CGI promoter methylation can occur in differentiation processes [325] and during the establishment of X chromosome inactivation [335], as it correlates with long-term stabilization of transcriptional silencing. It seems to be a mechanism to lock the gene in a repressed state, not the initiation event for silencing.

The reason why CGIs promoters are usually devoid of DNA methylation is not clear. Many mechanisms have been proposed: it seems unlikely that CGIs could be refractory to de novo methylation by DNMTs due to their DNA sequence; CGIs could be targeted by DNA demethylation mechanisms, but such demethylating activity in somatic tissues has not been identified yet; the presence in the CGIs of transcription factors or of specific histone modifications, as H3K4me3, could prevent DNMTs binding [336]. Additionally, it has been proposed that, since CGIs correlated with actively transcribed genes and since TSS of active genes are nucleosome-depleted, DNMTs are lacking the substrate for the de novo methylation, as their preferential substrate is nucleosomal DNA [337].

About 50% of CGIs are located in TSS proximity, while the other CGIs are the so-called "orphan CGIs". In these CGIs, which display high variability in the methylation pattern in different somatic cells and tissues, the chance of acquiring methylation is significantly higher than in the CGIs present at promoters, especially in the intragenic CGIs [338]. The role of this methylation is still under debate: it could be linked to the aforementioned role of DNA methylation in gene bodies, or it could regulate alternative splicing. Interestingly, some non-coding RNAs have CGIs in their promoters, which remain unmethylated, therefore putting forward the possibility that some of the so-called "orphan CGIs" could correspond to regulatory RNAs promoters.

As mentioned above, less then 70% of annotated gene promoters are associated with CGIs, but the remaining fraction of genes does not show CpG islands in the promoter region. DNA methylation in this context has not been completely clarified yet; substantial fluctuations of the DNA methylation pattern occur in these genes, where methylation seems to be more tissue-specific and dynamic compared to CGI promoter methylation [337].

The reverse process, DNA demethylation, is still poorly understood in mammals. DNA methylation can be achieved either passively, not methylating newly synthesized DNA after replication therefore "diluting" the methylated cytosines, or actively, in a replication-independent process. Passive demethylation is probably occurring during mammalian development in the maternal genome during pre-implantation growth [339], and it requires active cell division. In other tissues, both during embryonic development and in adult somatic cells there are evidences of replication-independent, active demethylation [307]. The mechanism leading to demethylation of 5mC is still under debate, but it is likely that different enzymatic pathways can be responsible for it. In plants, demethylation is achieved by a family of four DNA glycosylases, which first remove the base and then cleave the abasic site leaving a nick, which is repaired through Base Excision Repair (BER) mechanisms [340]. Glycosylases showing an homologous role have not been

identified in mammals yet, and the known mammalian glycosilases show very weak activity toward 5mC demethylation [341]. Deamination of 5mC to tymine resulting in T-G mismatch is also a candidate mechanism to mediate demethylation, since the mismatch could be recognized and resolved by BRE, restoring unmethylated cytosine [342]. Recently, the discovery of 5-hydroxymethylcytosine (5hmC) in mammalian genomes has suggested new possible mechanisms for DNA demethylation. The TET family of proteins are the enzymes involved in the oxidation of 5mC to 5hmC [343]; moreover, they are capable of iterative oxidation leading to the formation of 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC); these base modifications are detectable in the DNA of ES cells, but are at least one order of magnitude less represented compared to 5hmC. 5fC and 5caC could be recognized by different interactors compared to 5hmC; moreover, due to their chemical structure, they destabilize the N-glycosidic bond. Different mechanisms have been proposed to explain 5hmC as an intermediate of DNA demethylation. 5hmC could be passively diluted contributing to demethylation, or the oxidized methyl group could be actively removed in order to restore cytosine, or the modified nucleotide could be excised and repaired through DNA repair mechanisms [344]. It has been recently discovered that thymine DNA glycosidases (TDG), while they have no excision activity on 5mC or 5hmC, have robust in vitro base excision activity on 5fC and 5caC properly paired to G in duplex DNA, suggesting that they could mediate base excision of oxidized nucleotides [345]. In line with the hypothesized role of TDG in DNA demethylation, these proteins are necessary for correct embryonic development: TDG-null embryos show decreased expression of developmental regulators, with perturbed DNA methylation in regulatory sequences [346-347].

EPIGENETIC REGULATION OF TRANSCRIPTION BY NOTCH

The Notch signaling cascade appears to be quite simple, with no second messengers involved, however, the activation of the downstream genes is amazingly complex.

The transcription factor RBP-Jk plays a central role in transducing the Notch receptor cleavage signal to changes in gene expression, acting as either a transcriptional repressor or activator (**Fig.15**). When NICD migrates to the nucleus, it displaces the repressor complex associated to RBP-Jk through SKIP (Ski-interacting protein), which is stably associated to RBP-Jk in both the context of transcriptional activation and repression. SKIP is able to bind both the SMRT (silencing mediator of retinoid and thyroid hormone Receptors) corepressor complex and NICD, but this binding is mutually exclusive [348]. When NICD interacts with RBP-Jk, it acts as a bridging factor to form a ternary activator complex composed by RBP-Jk, NICD and Mastermind, a co-activator protein which is an integral part of the Notch signaling pathway [349-350]. Mastermind in turn recruits p300 [122, 351-353] or other known HATs, such as Pcaf or Gcn5, with cellular and tissue specificity [353-355] resulting in the formation of a multisubunit protein complex. Its combinatorial activity modulates the transcription at Notch-responsive promoters. The acetyltransferase complexes SAGA and Tip60 have been shown to be required for Notch and Mastermind activity for the correct wing development in Drosophila, probably enhancing the assembly of the Notch activator complex [356]. When the NICD signaling cascade is not activated, RBP-Jk interacts with distinct complexes of co-repressors: SKIP associates RBP-Jk and recruits SMRT/HDAC1 complex [357], therefore triggering transcriptional repression of Notch target genes. SHARP (SMRT and HDAC associated repressor proteins) interacts with RBP-Jk [358], acting as a "co-repressor hub" to recruit different complexes and contributing to the versatility of Notch regulated gene expression. The SHARP/RBP-JK complex have been shown to recruit Eto, a conserved nuclear protein not directly able to bind DNA, but exerting a negative regulation on transcription, interacting with corepressors as N-CoR and SMRT [359] and recruiting HDAC1, -2 and -3 [360]. CtBP and CtIP, owing to the CoREST/Lsd1 complex, which recruits on the chromatin HDAC1/2 [361], are also known to interact with RBP-Jk/SHARP [149, 362]. Of interest, CtBP is necessary to recruit Sirt1 to the repressive complex [363].

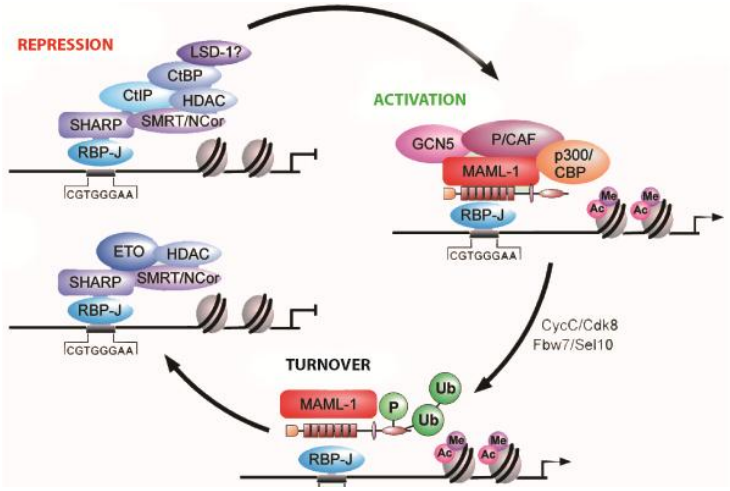


Figure 15. RBP-Jk mediates activation or repression of Notch signaling and interacts with different protein complexes in two opposite processes (adapted from [112]).

A variety of histone modifications have been associated to Notch promoters, as markers of active or inactive transcription (**Fig.16**).

H4K16 acetylation has been reported to play a dominant role on chromatin compaction and transcription, possibly disrupting a specific contact point between H4 and H2B. The enzyme responsible for this modification on Notch target genes promoters has not been identified yet, but Sirt1 has been reported to deacetylate H4K16, thus contributing to the silencing of Notch target genes [363]. In different models in Drosophila, H3K4 trimethylation has been reported to occur at Notch target gene promoters. These findings strongly support the possibility that histone methyltransferases are associated to the Notch-specific transcriptional complex when NICD is present, while demethylases are recruited in its absence [364-365]. The histone demethylase Lsd1, possibly recruited via SHARP-associated CtBP-Sirt complex [363], can associate with RBP-Jk [366] and can remove mono- and dimethylation on H3K4 [235]. Other demethylases, like Kdm5A have been reported to interact with RBP-Jk [367] and cooperate in the modulation of the chromatin environment at Notch target genes, as it happens in Drosophila [368]. The kinetics of the demethylation process is still unclear: the widely accepted model is that two

waves of demethylation take place, first involving Kdm5A/Lid (no longer needed after the removal of H3K4me3) and then Lsd1 [116]. Moreover, H3K4 trimethylation is also positively affected by the presence of H2B ubiquitination at Notch target gene promoters, therefore a functional crosstalk contributes to the complexity of the cellular responses to Notch activation [369].

The repressive mark H3K27 trimethylation the has also been described at Notch target gene promoters. A number of studies have established that PcG complexes are critical for both the proper function of Notch pathway and the expression of several Notch target genes [364, 370-371]. Studies in ES cells support the idea that, in order to activate Notch target genes transcription, H3K27me3 repressive marks are actively removed [372]. Moreover, a role for the PRC2 member Jarid2 has recently been postulated in the regulation of the Notch signaling during heart development. Jarid2 has been shown to repress Notch signaling pathway in embryonic heart development during the trabeculation process, possibly acting through histone lysine methylation [373].

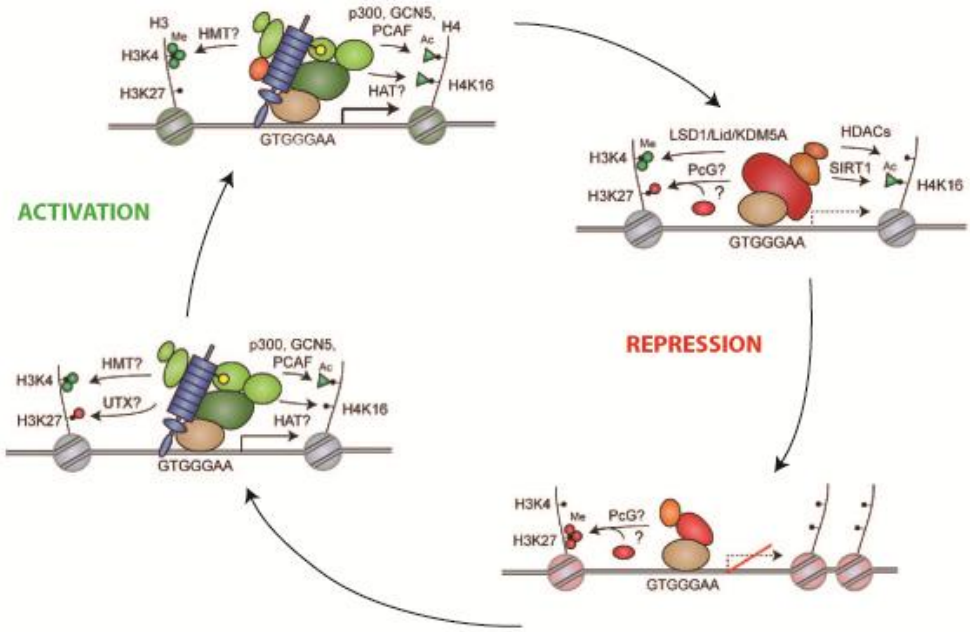


Figure 16. Histone modifications having a role in Notch signaling (adapted from [116]).

Chromatin remodeling factors are also involved in the control of Notch target gene expression. In vitro, it has been shown that a direct binding of SWI/SNF member Brahma to RBP-Jk occurs, both in the presence and absence of the NICD [374]. These results were supported by a genetic screen in Drosophila leading to the hypothesis that, once in the nucleus, NICD interacts with the Brahma complex, directly regulating its activity or bringing it to its target genes [375]. Moreover, in retinal stem cells/progenitors regulation, Brahma regulates progenitor commitment through attenuation of Notch signaling [376].

Another protein acting as a bridging factor between RBP-Jk and the BAF chromatin remodeling complex is Baf60c. This is a subunit of the SWI/SNF-like BAF complex, which plays a pivotal role during heart development in Zebrafish and in mouse in the regulation of Notch-mediated left-right asymmetry. Its role on Notch target genes promoters is to stabilize the interaction between activated Notch and RBP-Jk. [305].

Notch target gene silencing is also achieved through DNA methylation. In a model of dystrophic muscle, Notch1 is epigenetically silenced in response to elevated levels of TNFα and NF-kB, triggering the block of the regenerative potential of satellite cells [377]. In human B cell acute lymphoblastic leukemia (B-ALL), hypermethylation at the promoters of both Notch3 and Hes5 was associated with decreased H3K4 trimethylation and increase H3K27 trimethylation [378], therefore suggesting the formation of a transcriptional repressive chromatin environment at Notch target promoters.

ADENO-ASSOCIATED VIRAL VECTORS

Adeno-associated viruses

Adeno-associated viruses (AAV) were first described in 1965 by Atchinson as small contaminants of Adenovirus preparations, unable to generate a productive infection in the absence of helper viruses as Adenovirus or Herpesvirus [379]. They are classified in the family of Parvoviridae and in the genus of Dependoviridae. Almost

80% of the human population bears antibodies against AAV proteins, but, to date, no disease has been correlated to their infection.

AAVs are small icosahedral non-enveloped virions with a diameter of 25 nm. Their genome is a single-stranded DNA molecule of approximately 4.7 kb, with 145 nucleotide-long inverted terminal repeats (ITR) which form T-shaped hairpin structures at both ends of the genome, which are used as origins of replication and as primers for the second-strand DNA synthesis performed by the host DNA polymerase [380]. The double-stranded DNA intermediates are processed via a strand displacement mechanism, resulting in single-stranded DNA used for packaging and double-stranded DNA used for transcription [381]. Critical to the replication process are the Rep binding elements (RBEs) and a terminal resolution site (TRS) located within the ITRs. In addition to their role in AAV replication, the ITRs are also essential for AAV genome packaging, transcription, negative regulation under non-permissive conditions and site-specific integration (reviewed in [381-382]) (**Fig.17**).

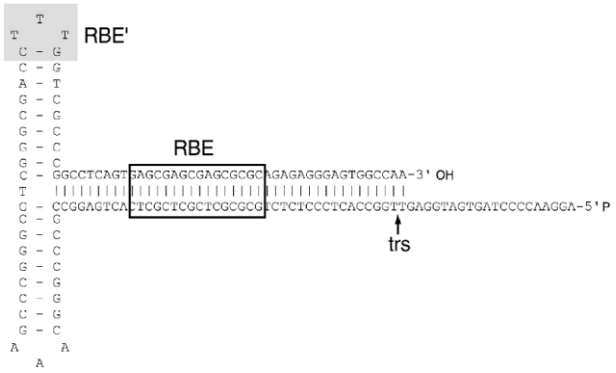


Figure 17. Secondary structure of the AAV2 ITR (adapted from [381]).

The AAV wild-type genome contains two ORFs encoding non-structural and structural proteins (**Fig.18**). The 5' ORF encodes *Rep* gene, coding for four different nonstructural proteins transcribed using two different promoters within the same gene (p5 and p19) through differential splicing. Rep78 and Rep68 positively regulate AAV gene expression in the presence of the helper virus. The proteins display site-

specific DNA binding activity (binding at the RBE) and site- and strand-specific endonucleases activity (nicking at the TRS to process the double-stranded intermediates) [383]. Rep52 and Rep40 are involved in the generation and accumulation of single-stranded viral DNA to be packaged into AAV capsids. All the four Rep proteins show helicase- and ATPase-activity [381]. The 3' ORF encodes the *Cap* gene, from which the three capsid proteins Vp1, Vp2, Vp3 are produced through different splicing sites and atypical translation start codons [384-385]. They differ from each other in the N-terminus and are assembled in a near spherical protein shell of 60 subunits with a ratio 1:1:10.

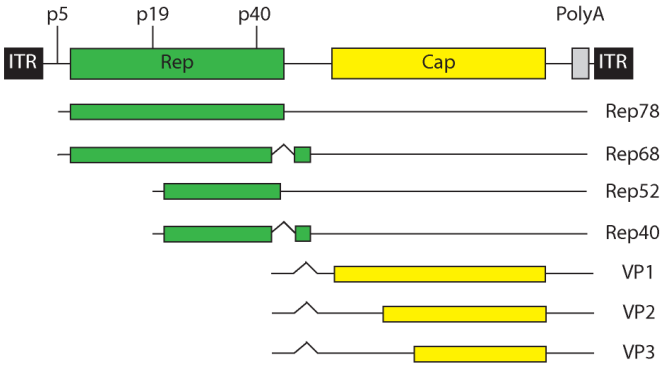


Figure 18. Adeno-associated virus genome organization.

AAV infection and viral life cycle

According to their serotype specificity, the virions recognize different receptors on the cell surface. The originally discovered and best characterized serotype is AAV2, which binds heparan sulphate proteoglycans (HSPG) of the cell surface [386] and at least six coreceptors, including $\alpha_V\beta_5$ integrins, fibroblast growth factor receptor 1, hepatocyte growth factor receptor, $\alpha_V\beta_1$ integrin and laminin receptor [387]. AAV3 also binds HSPGs; 3, O-linked and N-linked sialic acid are the known receptors for AAV4 and 5; the 37/67-kDa laminin receptor binds AAV8, AAV3 and AAV9 [388]; galactose is the primary receptor for AAV9 [389]; the epidermal growth factor receptor has been proposed as a coreceptor for AAV6 [390].

After docking to the membrane receptors, AAV2, for which more information is available, is endocytosed in clathrin-coated vesicles through a dynamin-dependent pathway; viral endocytosis is stimulated by binding of the virus to the receptor, which activates intracellular signaling pathways that stimulate receptor internalization [391]. Following internalization, endosomal acidification is necessary to induce conformational changes in the AAV capsid which expose the Vp1 and Vp2 N-termini outside the capsid, enabling the dissociation of the virus-receptor complex. The N-terminal of Vp1, buried inside the virion, contains a phopsholipase A2 domain necessary for the endosomal escape [392].

The virions are released into the cytosol after endosomal acidification but before their maturation to lysosomes [393]. The proteasome also plays a role in AAV2 trafficking, as its inhibition results in enhanced nuclear uptake of the virus [394].

After the release into the cytoplasm, defined sequences at the N-terminal part of Vp1 and Vp2 act as nuclear localization signals and trigger AAV genome transport into the nucleus, where it is uncoated and undergoes conversion from single- to double-stranded DNA intermediates (circular and linear). The mechanisms driving this conversion is not completely clear. It was hypothesized that the ITRs are used as primers during the double strand synthesis, but more recent evidence proposes that double-stranded DNA could result from the annealing of the single stranded DNA to a complementary copy [395].

In the presence of the Rep proteins, wild type AAV can also integrate its genome in the form of concatamers, preferentially in a region of human chromosome 19q13.3 called AAVS1 [396-397]. The AAVS1 site and AAV genome do not who show extensive regions of homology, with the exception of a short sequence containing the the same tetranucleotide repeat (GCTC), which is bound by Rep proteins [398]. This sequence, therefore, is believed to mediate Rep78 and Rep68 binding to the ITRs and to a Rep-specific nicking site (TRS), thus facilitating site-specific recombination between viral and cellular sequences. The phenomenon of site-specific integration into the human genome is a unique feature of AAVs and it is

considered attractive for a targeted stable transgene expression [399]. The minimal motifs necessary for AAV integration present in the AAVS1 locus, TRS and RBE, are located in the 5'UTR of the gene *Mbs85* (myosin binding subunit 85), also called *Ppp1r12c* (protein phosphatase 1 regulatory protein) [400].

When a cell bearing an integrated AAV genome is infected by the helper virus, a process of Rep-mediated excision of the viral genome from the host chromosome takes place, followed by a switch to the AAV lytic cycle. This event can only happen in the case of co-infection by Adenoviruses or Herpes simplex viruses, since the infection stimulates the cell to activate a set of genes with helper functions, allowing a permissive intracellular environment for AAV productive infection [381, 401] (schematic representation of AAV infection cycle in **Fig.19**).

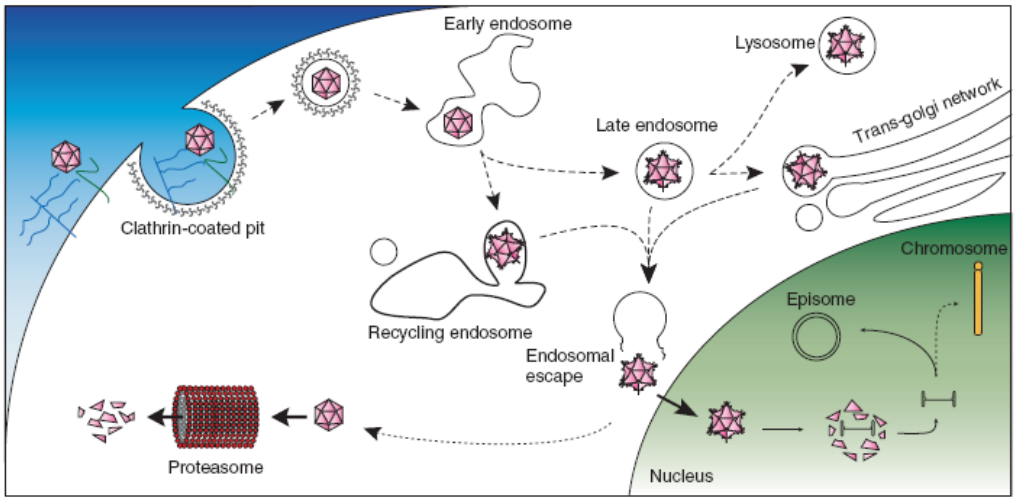


Figure 19. Cell entry and trafficking of Adeno-associated virus (adapted from [387]).

Adeno-associated viral vectors

Since 1984, AAVs have been considered as important tools for gene therapy, mainly because of their defective replication and their non-pathogenicity [402-403]. In the recombinant AAV (rAAV) genome, the *Rep* and *Cap* genes are replaced with the gene-expression cassette of interest, while the ITRs are maintained, since they are necessary for the correct viral packaging and protect the viral genome in the

infected cells. Initially, the rAAV particles were produced by transfecting packaging cells (HEK293 or Hela cells) with the rAAV genome and a construct carrying the viral *Rep* and *Cap* genes, followed by the infection of the cells with a helper Adenovirus (Ad) for an effective AAV infection. In this system, the rAAV underwent a normal lytic cycle, being rescued from the plasmid backbone and then packaged into particles [382]. Even if the contaminating Ad were eliminated by extensive purification and heat inactivation, the Ad contamination was a persistent problem [404]. To avoid it, in the late 1990s the infection with the Ad was substituted by the transfection with a plasmid construct containing a mini-Ad genome, capable of propagating rAAV in the presence of AAV Rep and Cap, but incapable of producing infectious Ad [405]. To improve the efficacy of the production, nowadays the packaging cells are transfected with two plasmid constructs, encoding for the therapeutic gene cassette flanked by ITRs and for Rep and Cap proteins and adenoviral proteins providing helper functions [406]. This system greatly increases efficacy, resulting in 10-fold higher titers of rAAV preparations compared to the previous method [404]. Twenty-four hours after the transfection, the cells are lysed and the rAAV vectors are purified by cesium chloride, iodixanol gradient centrifugation or chromatography [404, 407] (**Fig.20**).

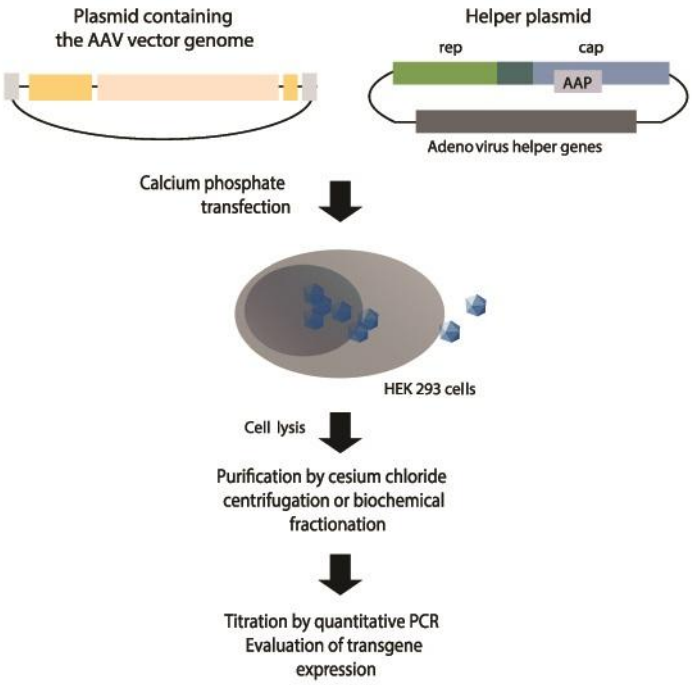


Figure 20. Recombinant AAV production system (adapted from [407]).

The standard constructs contain the ITRs and *Rep* and *Cap* genes of AAV2. To produce rAAVs with different tropism it is sufficient to use during the generation of the rAAVs a chimeric construct containing the *Rep* gene of AAV2 and the *Cap* gene of the serotype of interest [408].

In order to efficiently transduce different tissues in vivo, a variety of different serotypes are used, some of which naturally isolated and others produced artificially. Tissue specificity is due to the different surface receptors recognized by these vectors. AAV1 and -6 are efficient in the transduction of the skeletal muscle; AAV5, -7 and -8 efficiently transduce photoreceptors of the retina, while AAV5 and -4 transduce the pigment epithelium; AAV6 and -9 transduce the entire airway epithelium, while AAV5 transduction is limited to lung alveolar cells. AAV8 transduces liver, endocrine and exocrine pancreas; AAV5 and -9 are used for central nervous system gene transfer [381, 387].

For heart transduction, the first serotype used was AAV1, but additional studies demonstrated that capsid 9 confers the best cardiotropism in vivo in rodents [409-411], triggering a stable gene expression up to at least one year both in pups and in adults [412-413]. Interestingly, $1x10^{11}$ vg/mouse are able to transduce up to 80% of host cardiomyocytes (compared to 14% of heart transduction with AAV8). Vectors display high transduction efficiency either if injected intravascularly (tail vein, portal vein) or extravasculary (subcutaneously or intra peritoneum) in neonatal mice, while in the adult intra peritonal injection triggers a localized efficient transduction of the peritoneum and the diaphragm [409].

Recombinant AAVs are outstanding tools of the gene therapy of post-mitotic tissues, including the heart [409-410, 412, 414-415]. However, some caveats must be taken into consideration. In particular, AAV vectors are usually considered non immunogenic, based on animal studies, in which transduction of the liver was associated with the establishment of the immunological tolerance toward AAV vector and its transgene product [416-418]. However, results from a human clinical trial in which an AAV vector was injected into the liver for gene therapy of hemophilia B highlighted the possibility that transduction might lead to a cytotoxic T lymphocyte response towards the AAV capsid proteins, with consequent killing of the transduced hepatocytes [419-421]. Some strategies have been proposed to circumvent this problem, including the use of modified capsids [422], the transient immunosuppression of patients at the time of injection [423], the reduction of the dose of vector administered [419], or the introduction of vector into immunoprivileged sites when possible [424-425].

RESULTS

THE DECREASE OF CARDIOMYOCYTE PROLIFERATION AFTER BIRTH IS PARALLELED BY A REDUCTION IN NOTCH SIGNALING

In order to characterize the role of Notch pathway in the regulation of cardiomyocyte proliferation, we set up an in vitro culture model for rodent neonatal and adult cardiomyocytes. Neonatal cardiomyocytes were extracted from newborn rats (1-2 days old) and kept in culture up to one week, in order to reproduce in vitro their physiological exit from the cell cycle in the post-natal heart. Adult ventricular cardiomyocytes were extracted from 2-months-old adult female Wistar rats with Langendorff perfusion system and kept in culture for 2 days, as a physiological model of cardiomyocyte maturation (**Fig.21**).

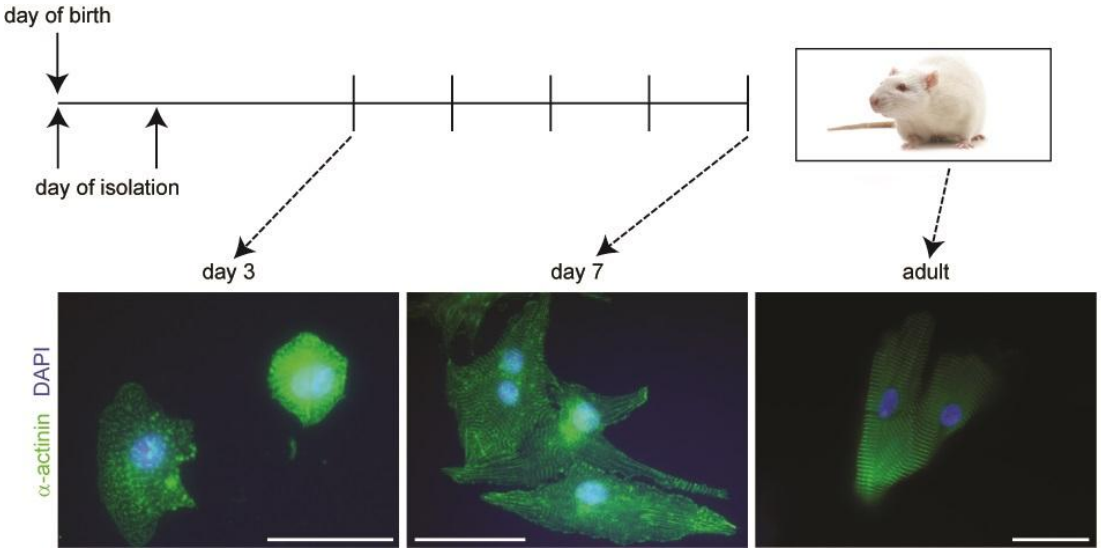


Figure 21. Experimental settings for the in vitro cardiomyocyte culture.
Cardiomyocytes are identified by the positivity to α-actinin staining. Scale bar 30 μm.

We first analyzed the proliferation rate of cardiomyocytes in our setting by the 5-bromo-2'-deoxyuridine (BrdU) incorporation assay. BrdU was administered for 20 hours before fixing the cells, which were subsequently processed for immunofluorescence analysis. We quantified the frequency of double positivity for the cardiomyocyte marker α-actinin and for BrdU: while after 3 days of culture almost 10% of the cardiomyocytes can incorporate BrdU, indicating that they are still cycling, this fraction of cells is significantly decreased at day 7, when approximately 3% of the cells are still positive for BrdU and become 0% in the adult cells, which were never detected BrdU+ (**Fig.22A**). We therefore stated, for our subsequent analyses, day 3 cardiomyocytes as proliferative cells and day 7 and adult cardiomyocytes as differentiated, non proliferating cells.

We next performed mRNA expression analysis for Notch1 and its target genes. We focused our attention on the *Notch1* gene, as Notch1 can autoregulate itself, and on the target genes previously shown to be regulated during cardiac development, *Hes1*, *Hey1* and *Hey2*, and *Cyclin D1*. We detected that all Notch1 target genes showed significant decrease in their expression between proliferative (day 3) and non proliferative (day 7 and adult) cardiomyocytes, paralleling their exit from the cell cycle (**Fig.22B**).

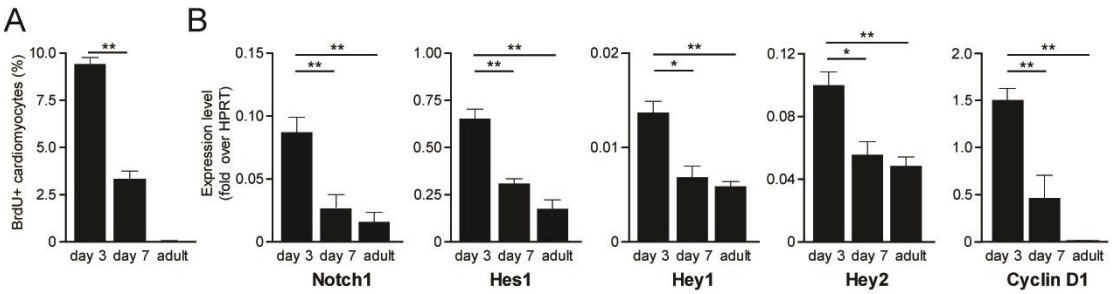


Figure 22. Suppression of Notch pathway upon cardiomyocyte terminal differentiation.

(**A**) Quantification of cardiomyocyte proliferation levels, assessed as the percentage of BrdU+, α-actinin+ cells, of neonatal rat cardiomyocytes at days 3 and 7 after isolation and adult rat cardiomyocytes. Shown are the mean±sem of at least three independent experiments. **: *P*<0.01.

(**B**) Transcription levels of Notch1 and its target genes in neonatal rat cardiomyocytes at days 3 and 7 after plating and in adult cardiomyocytes. Data are expressed to cellular HPRT mRNA levels. Shown are the mean±sem of at least three independent experiments. *: *P*<0.05; **: *P*<0.01.

To understand the molecular changes accompanying the decrease of Notch signaling, we performed chromatin immunoprecipitation (ChIP) experiments on day 3 (proliferative) and day 7 and adult (non proliferative) cardiomyocytes, in order to analyze the chromatin environment at Notch-responsive gene promoters. For each of the selected Notch1 target genes, sets of primers were designed and validated, mapping in correspondence of the gene transcription start sites (TSS). The housekeeping gene *GAPDH* promoter was used as a control (**Fig.23A**). We performed ChIP using antibodies detecting marks related to active chromatin (histone 3 pan-acetylation and histone 3 lysine 4 trimethylation) and to Polycomb-related repressive chromatin marks (histone 3 lysine 27 trimethylation, H3K27me3, hallmark of Polycomb Group protein 2 silencing and Ezh2 occupancy, being Ezh2 the only enzyme able to trimethylate lysine 27 on histone 3). When the cells were actively proliferating (day 3), Notch1 responsive genes were tagged by active chromatin marks, while in day 7 and adult non proliferative cells active chromatin marks decreased. No changes were detected in the *GAPDH* control promoter

(**Fig.23B** and **C**). The repressive chromatin marks showed an opposite behavior: H3K27me3 and Ezh2 occupancy were less present at day 3, when Notch1 signaling was active and cardiomyocytes were proliferating, while they were highly enriched in non proliferative day 7 and adult cells (**Fig.23D** and **E**). Collectively, these data indicate that, at Notch responsive promoters, chromatin moves from an active to an inactive state when the cells exit from the cell cycle.

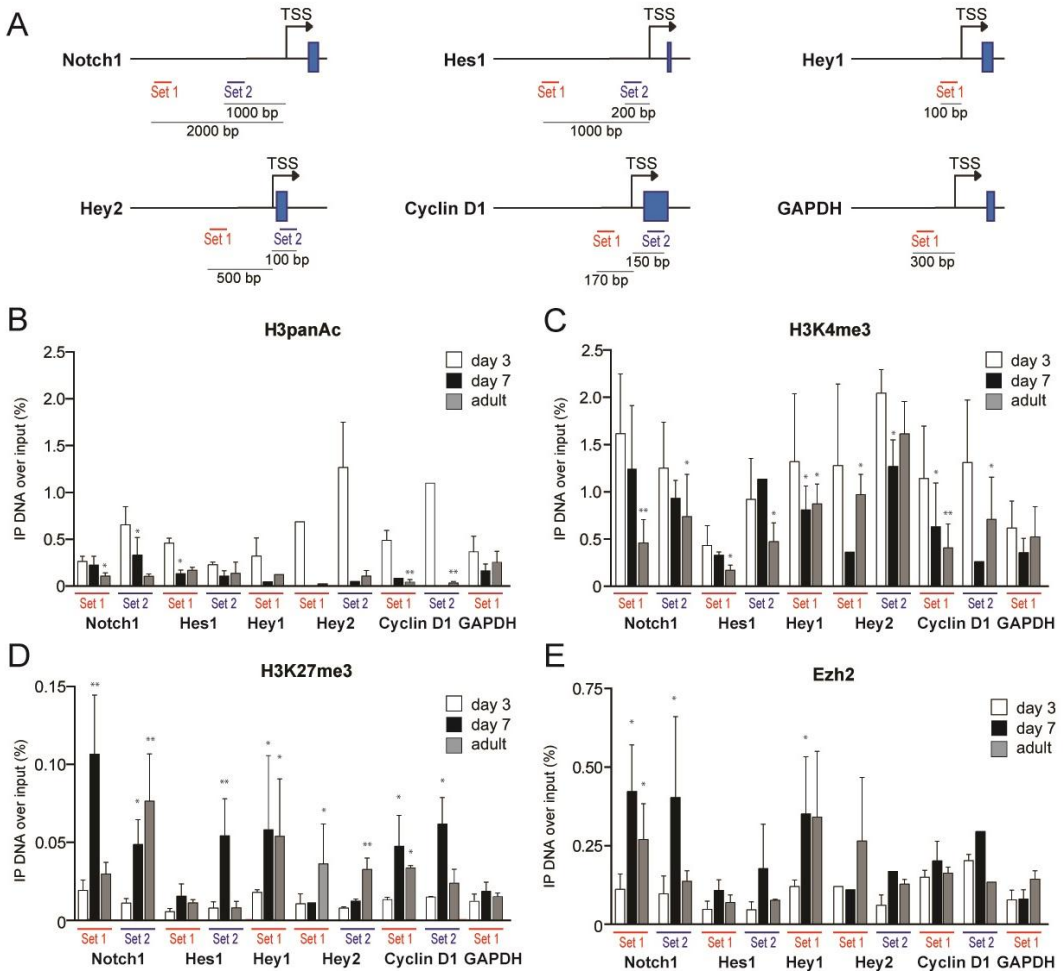


Figure 23. Chromatin modification at Notch target genes upon cardiomyocyte terminal differentiation.

(**A**) Localization of primer sets used for ChIP experiments in *Notch1* and its indicated target gene promoters.

(**B** - **E**) ChIP analyses of *Notch1* and target gene promoters on days 3 and 7 and adult cardiomyocytes using antibodies against active chromatin marks H3panAc, recognizing acetylated histone 3 (**B**) and H3K4me3, recognizing histone 3 lysine 4 trimethylation (**C**), and repressive chromatin marks H3K27me3, recognizing histone 3 lysine 27 trimehylation (**D**) and Ezh2 (**E**). Shown are the means±sem of at least three independent experiments. *: $P<0.05$; **: $P<0.01$ vs. day 3

We also confirmed that the change in Ezh2 occupancy at Notch target gene promoters was not a consequence of Ezh2 upregulation over time: the analysis of Ezh2 expression level in neonatal cardiomyocytes at days 3 or 7 and in adult cells did not show an increase in its expression over time (**Fig.24**).

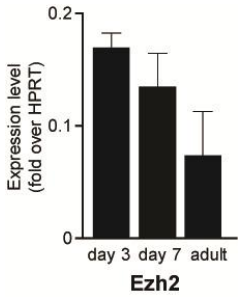


Figure 24. Analysis of Ezh2 expression level along cardiomyocyte differentiation. Transcription level of Ezh2 in neonatal rat cardiomyocytes at days 3 and 7 after plating and in adult cells. Data are expressed to cellular HPRT mRNA levels. Shown are the mean±sem of at least three independent experiments.

STIMULATION OF NOTCH PATHWAY BY AAV-MEDIATED GENE TRANSFER INDUCES NEONATAL CARDIOMYOCYTE PROLIFERATION IN VITRO

Given the correlation between Notch1 signaling and the extent of cardiomyocyte proliferation, we wondered whether the exogenous activation of Notch pathway could lead to an extended proliferative window of neonatal cardiomyocytes, also considering the previous results from our lab [196] and the well documented role of Notch1 in driving proliferation in the last phase of fetal life [121]. We took advantage of the excellent properties of the Adeno-associated viral vectors (AAV) in efficiently and stably transducing myocardial cells both in vitro and in vivo. We first confirmed the high efficiency of AAVs as tools to transduce in vitro cultured cardiomyocytes. Transduction was performed contextually to plating with an AAV6 vector encoding for EGFP at a m.o.i. of $1x10^4$ viral genome (vg) per cell. GFP+, α-actinin+ cardiomyocytes were then quantified at days 3 and 7 of culture. Transduction efficiency was approximately 30% at day 3 and increased until 40% at day 7 (**Fig.25A** for representative images and **25B** for quantification).

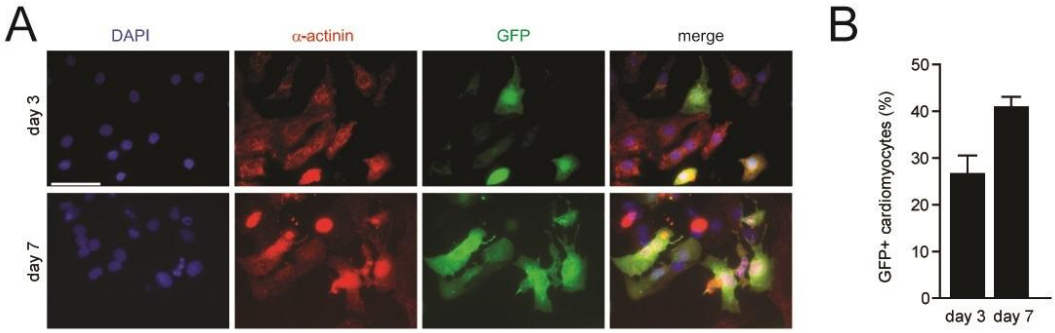


Figure 25. Transduction of neonatal cardiomyocytes by AAV serotype 6 vectors.
(**A**) Representative images of neonatal rat cardiomyocytes at days 3 and 7 of culture after transduction with AAV6-EGFP. Scale bar 100 μm.
(**B**) Quantification of neonatal cardiomyocyte positivity to GFP at days 3 and 7 after transduction with AAV6-EGFP.

In order to exogenously stimulate the Notch pathway, we designed two AAV vectors encoding for different players of the Notch1 signaling cascade. One vector encoded the constitutively active Notch1 intracellular domain (AAV6-N1ICD), while the other vector coded for the soluble form of its ligand Jagged1 (AAV6-sJ1), which is secreted and can act as a soluble cytokine in the medium, activating Notch signaling in a contact-independent manner (**Fig.26A**). We transduced neonatal cardiomyocytes at a m.o.i. of $1x10^4$ vg per cells contextually to plating, and we first analyzed transgene expression at days 3 and 7. Both the vectors were efficiently expressed and the transgene mRNAs were detectable at both days 3 and 7 (**Fig.26B**).

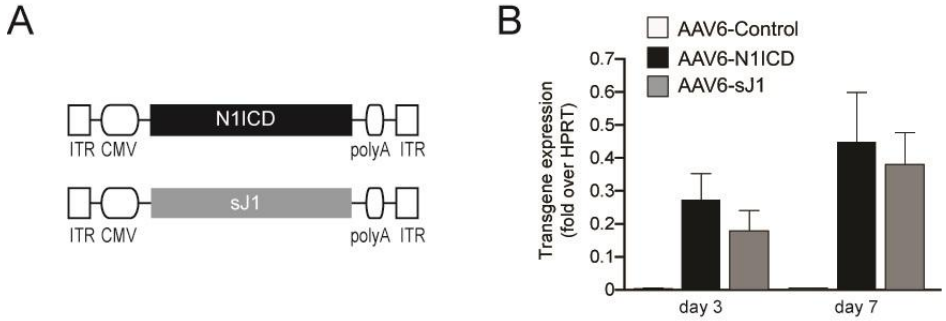


Figure 26. AAV6-N1ICD and AAV6-sJ1 are efficiently expressed in neonatal cardiomyocytes.

(**A**) Schematic representation of AAVs encoding for Notch1 intracellular domain (N1ICD) or a soluble form of Jagged1 (sJ1). ITR: AAV inverted terminal repeat. CMV: cytomegalovirus immediate early promoter. Poly A: polyadenylation site.

(**B**) Expression level of AAV6 transgenes in days 3 and 7 rat neonatal cardiomyocytes. Data are expressed to cellular HPRT mRNA levels. Shown are the means±sem of at least three independent transduction experiments.

While AAV6-sJ1-mediated signaling acts through the endogenous Notch1 pathway, AAV6-N1ICD should bypass the endocellular Notch1-processing steps. Therefore, in order to verify that the AAV6-N1ICD actually activates the canonical Notch signaling cascade in neonatal cardiomyocytes, we performed co-immunoprecipitation experiments to demonstrate that a physical interaction between the transgene molecule and RBP-Jk occurs. RBP-Jk was detected by Western blot in the protein complex immunoprecipitated from the whole cell lysate using a N1ICD-Myc-tag

specific antibody, demonstrating that the transgene acts through the canonical Notch1 pathway (**Fig.27**).

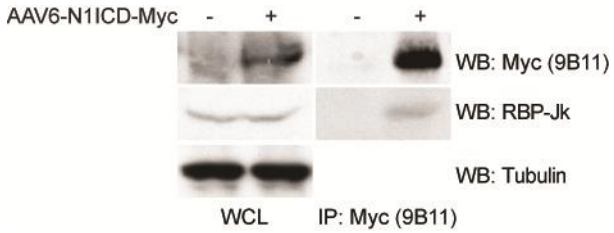


Figure 27. Transduced N1ICD binds endogenous RBP-Jk protein in cardiomyocytes.
Detergent solubilized proteins from rat cardiomyocytes transduced with AAV6-N1ICD were immunoprecipitated with anti-Myc tag antibody and analyzed by Western blot using anti-RBP-Jk antibody.

We then analyzed the effect of Notch1 pathway stimulation as an inducer of neonatal cardiomyocyte proliferation, evaluating the BrdU incorporation capacity of transduced cells in culture. In the case of AAV6-N1ICD-transduced cardiomyocytes, the fraction of α-actinin+, BrdU+ cells was 14.5% at day 3 and 18.7% at day 7, to be compared with 9.4% and 4.1% in cells transduced with a control AAV6 vector. Instead, proliferation did not increase at day 3 in cells transduced with AAV6-sJ1, however it was remarkably higher than the control at day 7 (15.7% proliferating cells; $P<0.01$; **Fig.28A** for representative images, **28B** for quantification). These different kinetics most likely reflect the requirement for soluble Jagged1 to accumulate in the extracellular environment to cluster and activate the downstream signaling.

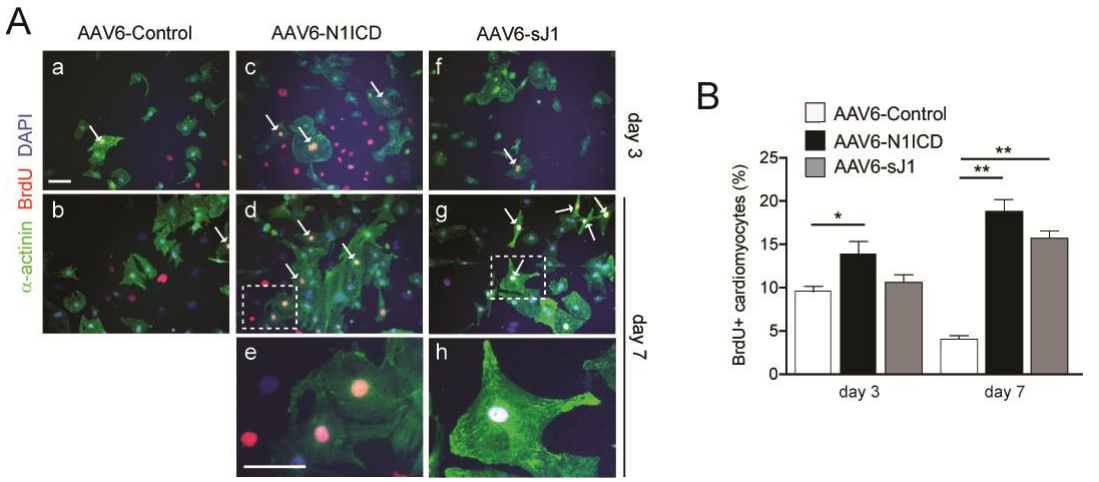


Figure 28. Stimulation of Notch pathway through AAVs expands the proliferative potential of neonatal cardiomyocytes.

(**A**) Representative images of cultured neonatal rat cardiomyocytes at days 3 and 7 of culture after transduction with a control AAV6 (**a** and **b** respectively), AAV6-N1ICD (**c** and **d**) or AAV6-sJ1 (**f** and **g**). Panels **e** and **h** show magnifications of the boxed areas in **d** and **g**, respectively. White arrows point at cardiomyocyte nuclei positive for BrdU. Scale bar 100 μm (**a**, **b**, **c**, **f**, **d** and **g**); 30 μm (**e** and **h**).

(**B**) Quantification of neonatal cardiomyocyte proliferation at days 3 and 7 after transduction with a control AAV6 vector, AAV6-N1ICD or AAV6-sJ1. Shown are the means±sem of at least three independent experiments. *: $P<0.05$;**: $P<0.01$.

BrdU is incorporated in the nascent DNA during the S phase of the cell cycle, therefore BrdU positivity is a marker of DNA replication, not necessarily of cell proliferation. To demonstrate that Notch1 stimulation also triggers mitosis and cytokinesis, we performed additional immunofluorescence staining to detect positivity for histone 3 serine 10 phosphorylation (a marker of mitosis, **Fig.29A** for representative images at day 7 and **29B** for quantification) and for the localization of Aurora B kinase in midbodies (showing cytokinesis, **Fig. 29C** for representative images at day 7 and **29D** for quantification). At day 7, both vectors were able to significantly increase the number of cardiomyocytes positive for both markers of cell cycle progression, showing that Notch pathway stimulation is able to lead cardiomyocytes to complete the cell cycle.

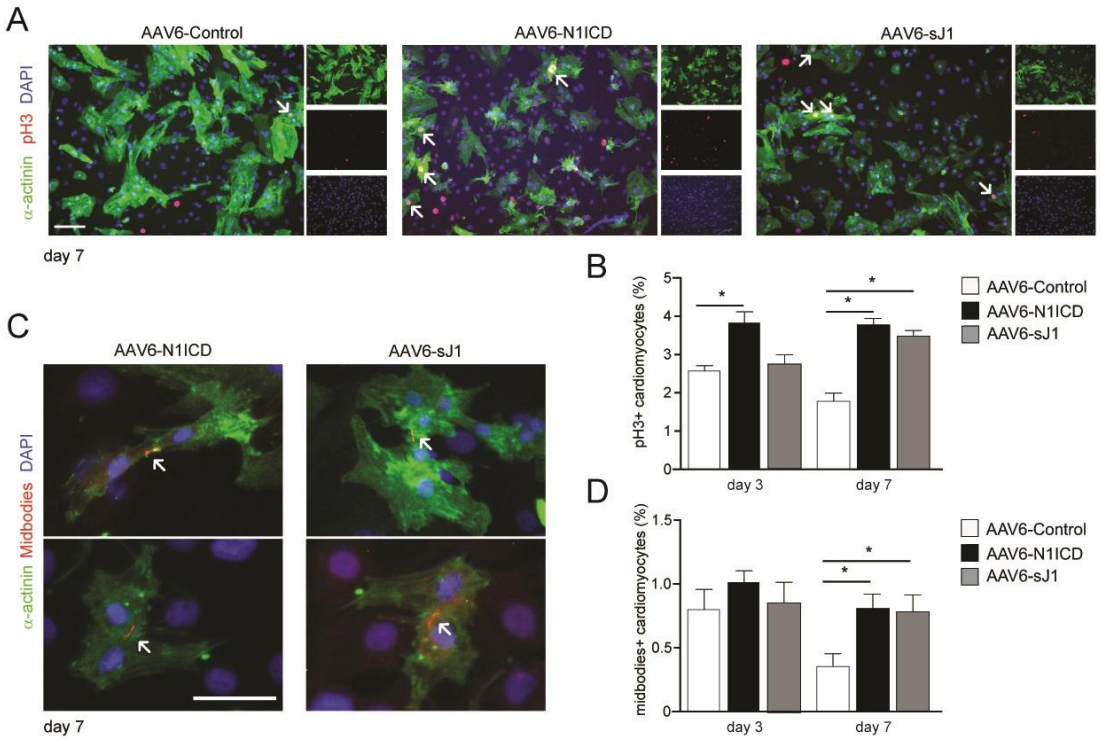


Figure 29. AAV6-N1ICD or AAV6-sJ1 transduction increases mitosis and cytokinesis of neonatal cardiomyocytes.

(**A**) Representative images of rat neonatal cardiomyocytes transduced with the indicated vectors and analyzed, at day 7, by immunofluorescence against histone 3 phosphorylated on serine 10 (pH3; red). Cardiomyocytes are stained in green with an anti-α-actinin antibody; nuclei are visualized in blue by DAPI. The leftmost panels show image splitting. Arrows point at mitotic cardiomyocytes. Scale bar 100 µm.

(**B**) Quantification of pH3+ cardiomyocytes. Shown are the means ± sem of at least three independent experiments. *: $P<0.05$.

(**C**) Representative images of rat neonatal cardiomyocytes transduced with the indicated vectors and analyzed, at day 7, by immunofluorescence against Aurora B (red). Cardiomyocytes are stained in green with an anti-α-actinin antibody; nuclei are visualized in blue by DAPI. Arrows point at midbodies. Scale bar 30 µm.

(**D**) Quantification of midbodies in cardiomyocytes. Shown are the means ± sem of at least three independent experiments. *: $P<0.05$.

To better characterize the Notch1 pathway signaling cascade upon transduction with AAVs, we performed a series of experiments in neonatal cardiomyocytes in the presence or absence of the γ-secretase inhibitor DAPT, which blocks the last step of processing of the endogenous Notch receptor, preventing its release into the cytoplasm. This experimental strategy allowed us to distinguish between the Notch signaling downstream the endogenous receptor, blocked by DAPT treatment, and the signaling activated by the exogenously expressed AAV6-N1ICD, directly acting in the nucleus, therefore insensitive to γ-secretase inhibition.

Neonatal cardiomyocytes, transduced with either AAV6-N1ICD or AAV6-sJ1 were transfected, at days 2 or 6, with a plasmid expressing the Firefly Luciferase under the control of either the Hes1 promoter (**Fig.30A,** panels a and b) or of a synthetic promoter containing 4xRBP-Jk responsive sites (**Fig.30A,** panels c and d); Luciferase activity was measured 24 hours after transfection. Both AAVs were able to induce transgene expression at day 7, while only AAV6-N1ICD significantly stimulated activity at day 3, paralleling the different vector kinetics. DAPT treatment was effective in abolishing AAV6-sJ1 mediated Hes1-transcription, while it was largely ineffective on AAV6-N1ICD-driven signaling. These results show that the AAV6-sJ1 molecule activates the endogenous intracellular Notch1 signaling cascade, while the AAV6-N1ICD protein directly acts on the transcriptional machinery to activate the Notch1 pathway. A superimposable effect of DAPT was found by analyzing cardiomyocyte proliferation upon AAVs transduction: only AAV6-sJ1-transduced cardiomyocytes showed a marked decrease in BrdU positivity at day 7 in the presence of DAPT (**Fig.30B**), showing that the effect driven by AAV6-N1ICD mostly depends on the exogenously expressed protein rather than on the endogenous receptor.

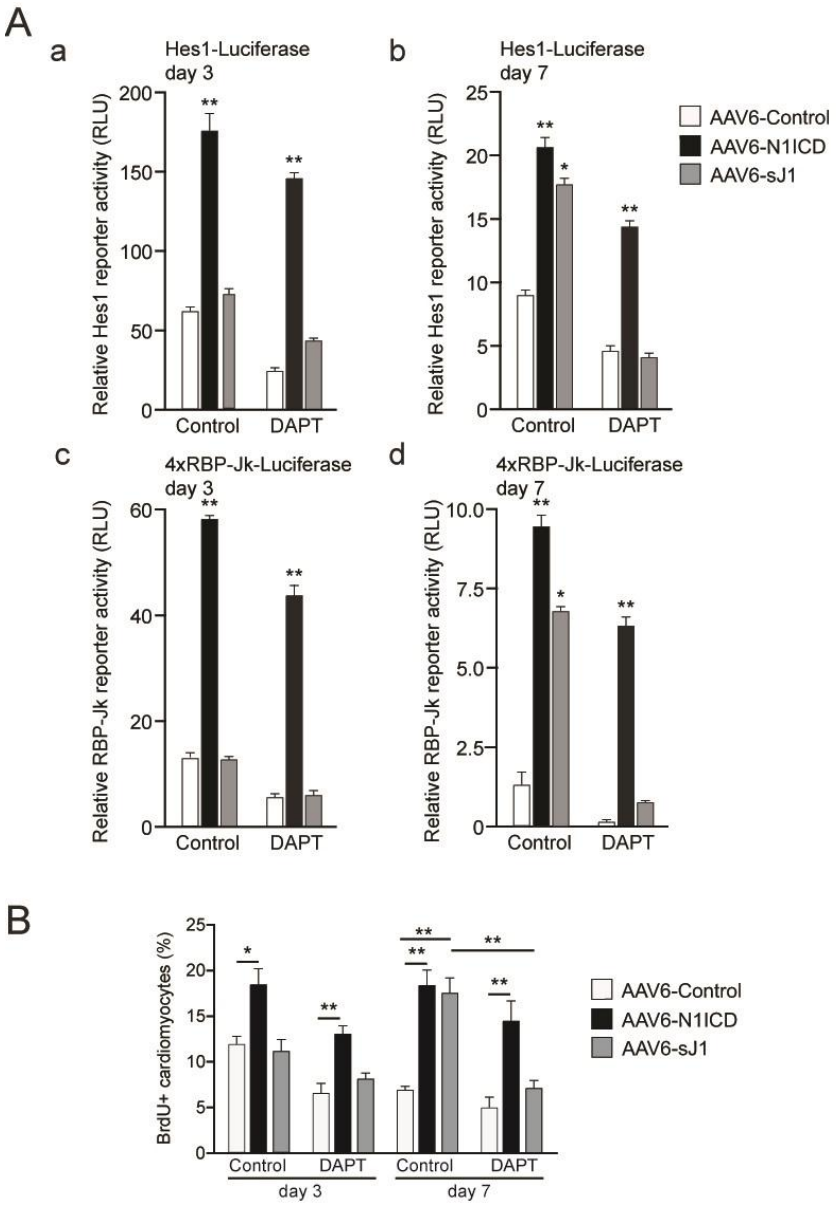


Figure 30. The effects of AAV6-N1ICD are not mediated by endogenous Notch1 receptor signaling.

(**A**) Neonatal rat cardiomyocytes were transfected with either a Hes1-Luciferase or a 4xRBP-Jk-Luciferase reporter constructs (panels a-b and c-d respectively) and analyzed at days 3 or 7 (panels a-c and b-d respectively). Transfection efficiency was standardized by co-transfecting a constitutively expressed Renilla Luciferase reporter. The histograms show mean±sem; n = 6; *: $P<0.05$; **: $P<0.01$ vs. control.

(**B**) Quantification of BrdU incorporation in neonatal rat cardiomyocytes at days 3 and 7 after transduction with either AAV6-N1ICD or AAV6-sJ1, in the presence of DAPT or in control condition. Shown are the means ± sem of at least three independent experiments. *: *P*<0.05; **: *P*<0.01 vs. control.

To verify whether Notch target gene expression is stimulated by AAV transduction, we analyzed their expression levels in day 3- and day 7-cultured neonatal cardiomyocytes transduced at day 0 with either AAV6-N1ICD or AAV6-sJ1. These levels were found to parallel the trend of cardiomyocyte proliferation, with AAV6-N1ICD already acting at day 3 on some targets, while both vectors were able to significantly stimulate target gene expression at day 7 (**Fig.31**). Activation of the Notch pathway by AAV6-N1ICD at days 3 and 7 and AAV6-sJ1 at day 7 also resulted in the increase of endogenous Notch1 expression, consistent with the existence of a positive feed-back loop by which Notch activates its own gene expression [426].

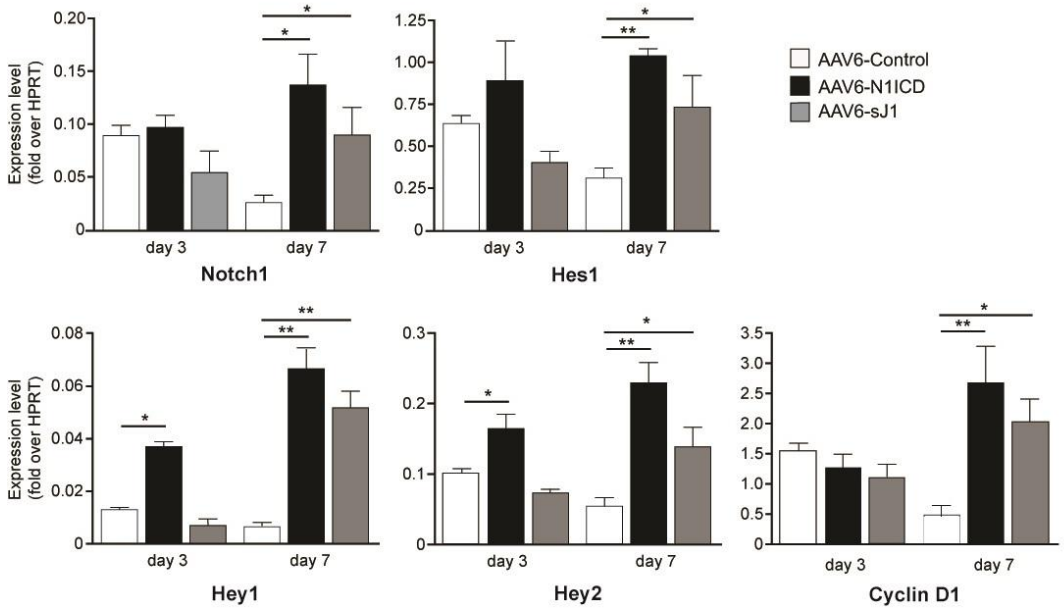


Figure 31. AAV-mediated transduction stimulates Notch1 target gene transcription.

Transcription level of Notch1 and its target genes in neonatal rat cardiomyocytes analyzed at days 3 and 7 after AAV6 vector transduction. Shown are the means±sem of at least three independent experiments. *: *P*<0.05;**: *P*<0.01.

We then characterized the chromatin environment at the Notch responsive promoters in the presence of Notch pathway constitutive stimulation. Chromatin immunoprecipitation experiments were performed in neonatal cardiomyocytes cultured for 3 or 7 days and transduced contextually to plating with either AAV6-N1ICD or AAV6-sJ1. In cells transduced with either of the two Notch-stimulating vectors, the chromatin at the analyzed Notch1 responsive genes was significantly more acetylated at day 7 than in control-treated cardiomyocytes (**Fig.32A**). On the contrary, the amount of chromatin marked by H3K27me3 and the Ezh2 promoter occupancy, which increased in control conditions at day 7, remained stable in the transduced cells (**Fig. 32B** and **32C** respectively).

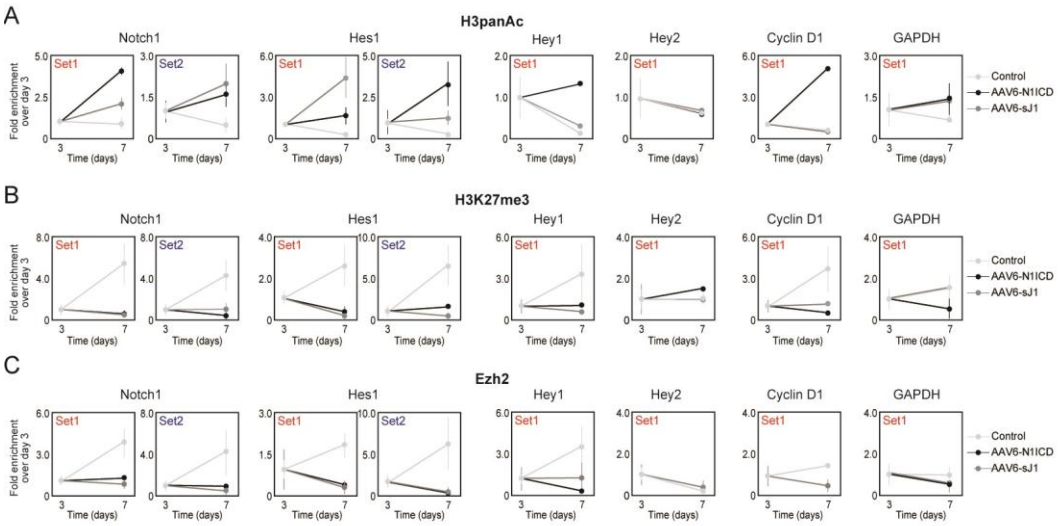


Figure 32. AAV-mediated Notch activation maintains chromatin open at Notch target gene promoters.

(**A** - **C**) ChIP analyses for *Notch1* and its target gene promoters in control-infected cardiomyocytes (light grey line), or cardiomyocytes transduced with AAV6-N1ICD (black line) or AAV6-sJ1 (dark grey line) at days 3 and 7 after transduction. Values are normalized over the results at day 3. Antibodies were against H3panAc (**A**), H3K27me3 (**B**) and Ezh2 (**C**). Shown are the means±sem of at least three independent experiments.

We also verified that the Notch pathway stimulation was not affecting Ezh2 expression, to rule out the possibility that the low Ezh2 occupancy at Notch responsive promoters was a mere consequence of a decreased expression or repressed transcription of Ezh2 induced by Notch activation. RT-PCR experiments confirmed that the levels of the endogenous Ezh2 did not change upon AAV transduction neither in day 3 nor in day 7 cardiomyocytes, therefore confirming that the delayed recruitment of Ezh2 at Notch target gene promoters when Notch pathway was exogenously activated by AAVs was not induced by a reduction in Ezh2 expression level (**Fig.33**).

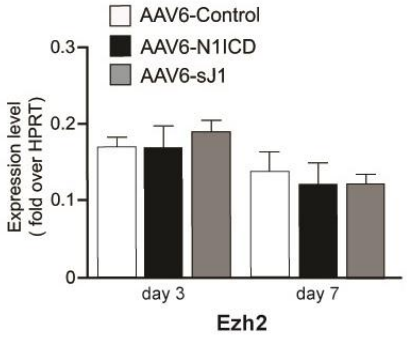


Figure 33. Analysis of Ezh2 expression level after exogenous Notch pathway activation.

Transcription levels of Ezh2 in neonatal rat cardiomyocytes analyzed at days 3 and 7 after AAV6 vector transduction. Data are expressed to cellular HPRT mRNA levels. Shown are the mean±sem of at least three independent experiments. *: $P<0.05$; **: $P<0.01$.

Recent evidences have highlighted an important role of Polycomb in the regulation of cardiomyocyte proliferation during heart development [427-428]. To further prove the role of PcG-mediated transcriptional silencing at Notch responsive genes, we treated cardiomyocytes with the specific Ezh2 inhibitor GSK126. These experiments were performed in various conditions: when the cells were analyzed at day 3, the drug was administered for 2 days before fixation and DMSO was used as a control (panel A, condition a-a'-b). For the analysis at day 7, the drug was added for 2 days before fixation (condition d) or for 4 days before fixation, therefore inhibiting Ezh2

action between days 3 and 7 of culture (condition e), and DMSO was added as a control. Since GSK126 efficacy was demonstrated only in a panel of lymphoma cell lines [429], we needed to characterize its effect and determine the working conditions in our experimental settings. We therefore analyzed the efficacy of GSK126 in inhibiting Ezh2 action on neonatal cardiomyocytes by checking the total level of H3K27me3 in our experimental conditions. In Western blot experiments, we detected a decrease of the total level of H3K27me3 upon GSK126 treatment for either 2 or 4 days, demonstrating its effect on cultured cardiomyocytes (**Fig.34A** and **B**).

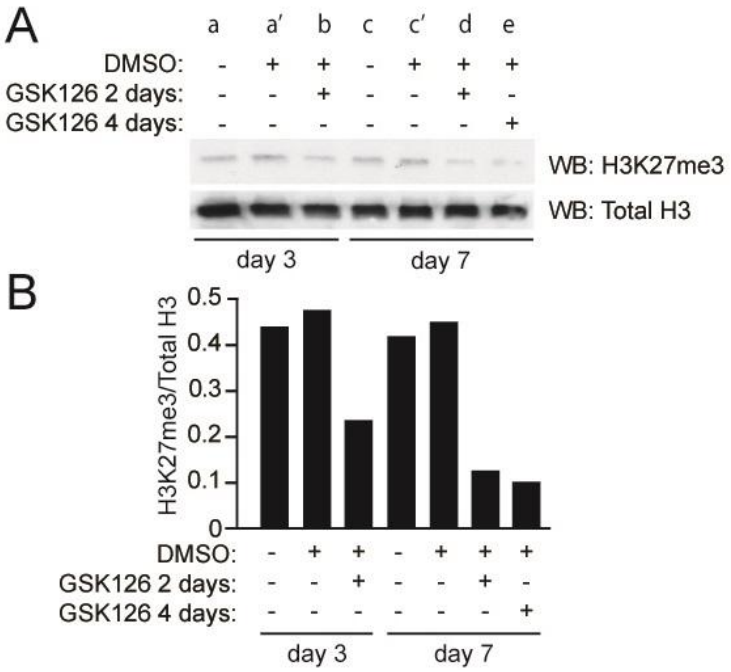


Figure 34. The Ezh2 inhibitor GSK126 is able to reduce the total level of H3K27me3.

(**A**) Western blot analysis of H3K27 trimethylation levels in day 3 and day 7 cardiomyocytes in the experimental conditions as indicated (a′ and c′ correspond to the DMSO vehicle control). Total Histone H3 is shown as a loading control.

The blot is representative of at least three independent replicates.

(**B**) Quantification of the blot in panel A.

We then analyzed the effect of Ezh2 inhibition on cardiomyocyte proliferation. At day 3, the addition of the drug had no significant effect on proliferation, nor it acted synergistically with AAV6-N1ICD to boost proliferation (**Fig.35**, panels a and b). This can be explained by the fact that, at day 3, Ezh2 is not strongly active at Notch-related promoters. When GSK126 was added for 2 days before fixing the cells at day 7, the inhibition of Ezh2 activity had a proliferative effect similar to the stimulation of the Notch pathway (**Fig.35**, panel d), but no strong synergistic effect was detected in the presence of AAV6-N1ICD. When cultured cardiomyocytes were treated for 4 days with GSK126 between days 3 and 7, the inhibition of Ezh2 in the time frame when the chromatin at Notch target genes passes from active to repressive transcriptional state synergized with Notch pathway stimulation mediated by AAV6-N1ICD transduction, reaching 22.2% of BrdU+ cardiomyocytes (**Fig.35**, panel e), indicating that inhibition of the establishment of a repressive chromatin environment and stimulation of Notch pathway can cooperate to promote cardiomyocyte proliferation.

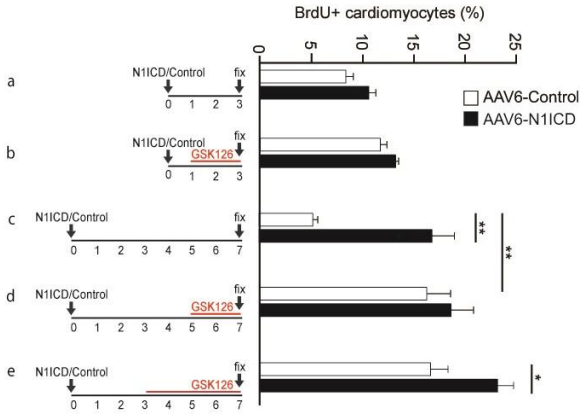


Figure 35. The Ezh2 inhibitor GSK126 synergizes with Notch pathway stimulation in promoting cardiomyocyte proliferation.

Quantification of neonatal cardiomyocyte proliferation (BrdU incorporation) in the indicated experimental conditions.

a-b: analysis at day 3, after AAV6-Control or AAV6-N1ICD transduction at day 0 and GSK126 treatment as indicated. *c-e*: analysis at day 7. Shown are the means±sem of at least three independent experiments. *: $P<0.05$; **: $P<0.01$.

AAV-MEDIATED NOTCH PATHWAY ACTIVATION DOES NOT STIMULATE HEART REGENERATION IN ADULT MICE AFTER MYOCARDIAL INFARCTION

Since these results indicate that gene-transfer-mediated Notch pathway stimulation can significantly expand the proliferative capacity of neonatal cardiomyocytes, maintaining an open chromatin conformation at the Notch-responsive genes and thus sustaining high levels of transcription, we decided to test if Notch pathway stimulation could be effective in vivo to promote tissue regeneration after myocardial infarction (MI). To do so, we performed myocardial infarction through the ligation of the left anterior descending coronary artery in adult CD1 mice. The animals were contextually injected with AAV9 vectors encoding N1ICD or sJ1 in the peri-infarct zone or with AAV9-Control vector (**Fig.36**).

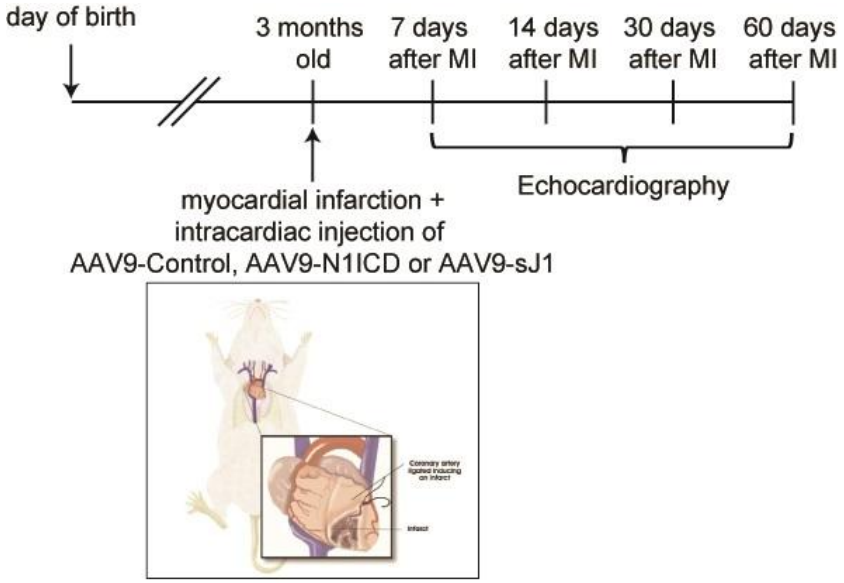


Figure 36. Scheme of the in vivo experiment.

The efficiency of transduction was assessed at DNA level through competitive PCR at days 7, 14, 30, 60 after transduction. The viral genome abundance was highest at

day 7, decreased at day 14, remaining constant until 60 days after transduction (**Fig.37A**). The transgene expression efficiency was evaluated at both mRNA and protein levels (**Fig.37B** and **37C** respectively), proving that the transgenes were detectable throughout the length of the experiment.

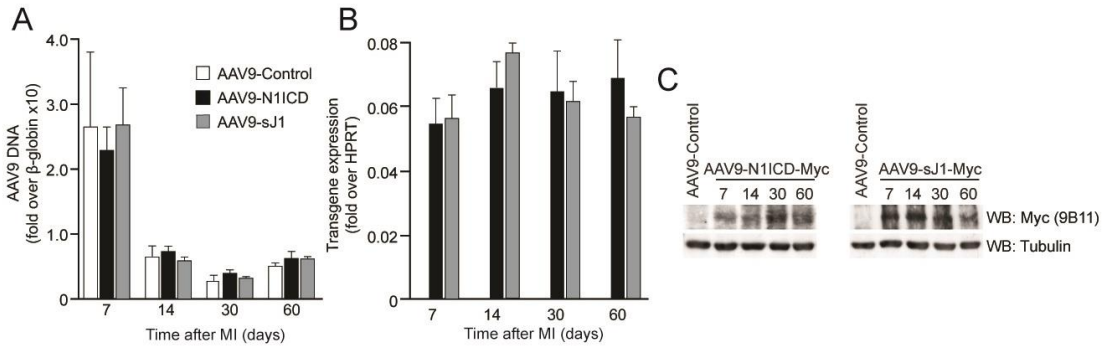


Figure 37. AAV9-mediated transgene expression is effective till 60 days post transduction.

Quantification of neonatal viral genomes (**A**), levels of mRNA transgene expression (**B**) and of protein transgene expression (**C**) in mouse hearts at different times after intracardiac injection of AAV9-N1ICD, AAV9-sJ1 or AAV9-Control, the last containing an empty MCS (n=3 per group).

Contrary to our expectation, when we analyzed the functional parameters of transduced animals through echocardiography, we could not detect any significantly improved cardiac performance in mice transduced with either vector. In particular, we did not appreciate any improvement of ejection fraction (LVEF, **Fig.38A**), fractional shortening (LVFS, **Fig.38B**), anterior wall thickening (LVAWT, **Fig.38C**), systolic and diastolic anterior wall thickness (LVAWT-s and LVAWT–d, **Fig.38D** and **38E** respectively) in mice transduced with AAV9-N1ICD, while a slightly better outcome was detected in AAV9-sJ1 treated animals, but this beneficial effect in any case did not reach statistical significance. The absence of a regenerative response was confirmed also at the histological level. After Masson trichrome staining to visualize the scar area in heart sections, we could not detect any reduction in the scar size in the transduced animals (**Fig.38F** for representative images, **Fig.38G** for quantification).

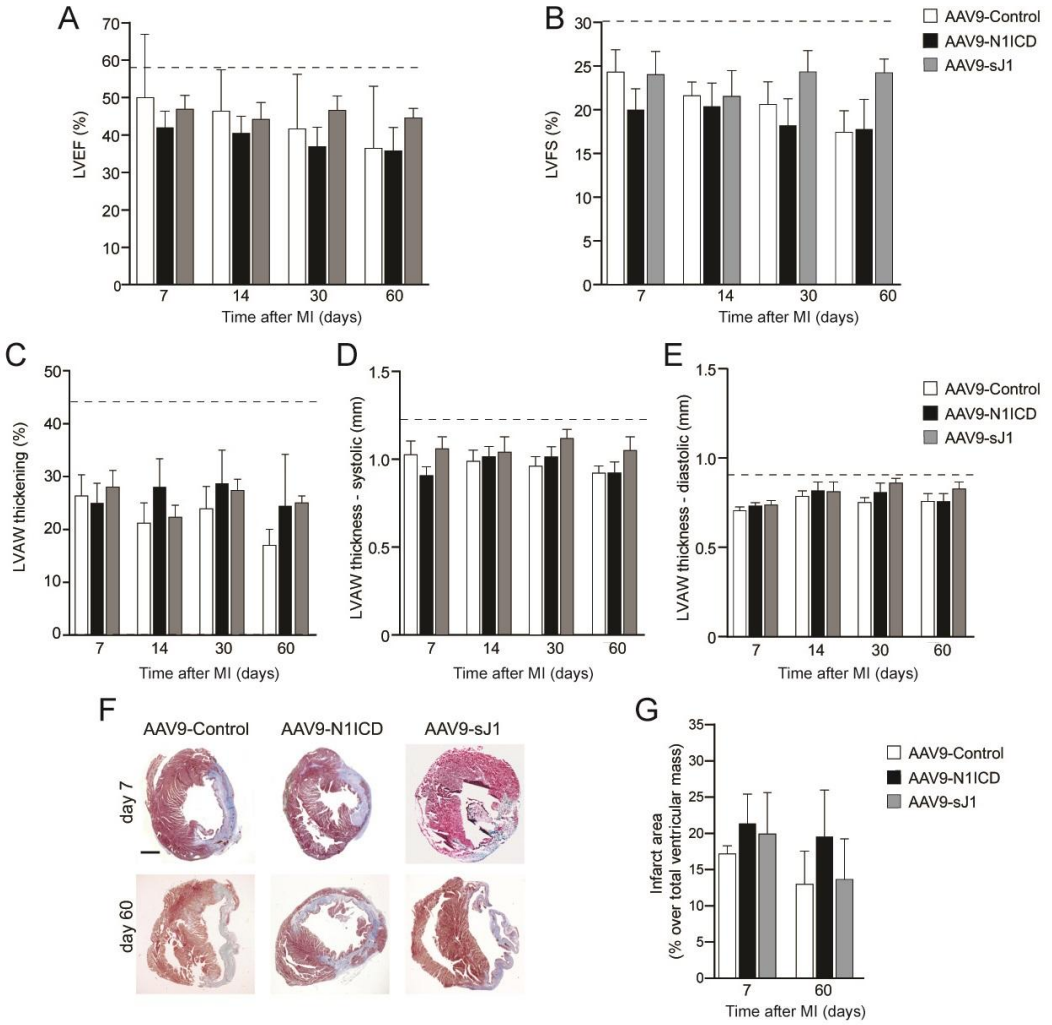


Figure 38. Stimulation of the Notch pathway fails to improve myocardial function and to induce repair after myocardial infarction.

(**A-E**) Evaluation of cardiac function at different times after MI in mice transduced with AAV9-Control, AAV9-N1ICD or AAV9-sJ1. The analyzed parameters are left ventricular ejection fraction (**A**), fractional shortening (**B**), anterior wall thickening (**C**), systolic anterior wall thickness (**D**) and diastolic wall thickness (**E**). *n*=10-12 per group; *P*= n.s. Dashed lines indicate the average value of non-infarcted animals.

(**F** and **G**) Representative images of Masson trichrome staining (**F**) and quantification of infarct size (**G**) at 7 and 60 days after MI in adult mice injected with AAV9-Control, AAV9-N1ICD or AAV9-sJ1. Scale bar 1 mm.

AAV-MEDIATED NOTCH PATHWAY ACTIVATION DOES NOT INDUCE ADULT CARDIOMYOCYTE PROLIFERATION IN VITRO

To understand the cause of the lack of action of the Notch pathway activation in vivo in the adult mice, we moved back to an in vitro system of adult cultured cardiomyocytes and tested the effect of Notch stimulation on these cells. We first needed to assess whether the AAV6 serotype was able to transduce adult cardiomyocytes as efficiently as neonatal cells in vitro. Adult ventricular cardiomyocytes were extracted by the Langendorff perfusion system, transduced with AAV6-EGFP and analyzed for the positivity for α-actinin and GFP. In the absence of serum, adult cardiomyocytes were efficiently transduced by AAV6 reaching 60% of GFP positivity. When cultured with serum, which is required for their proliferation, transduction efficiency was around 40% (**Fig.39A** for representative images, **Fig.39B** for quantification).

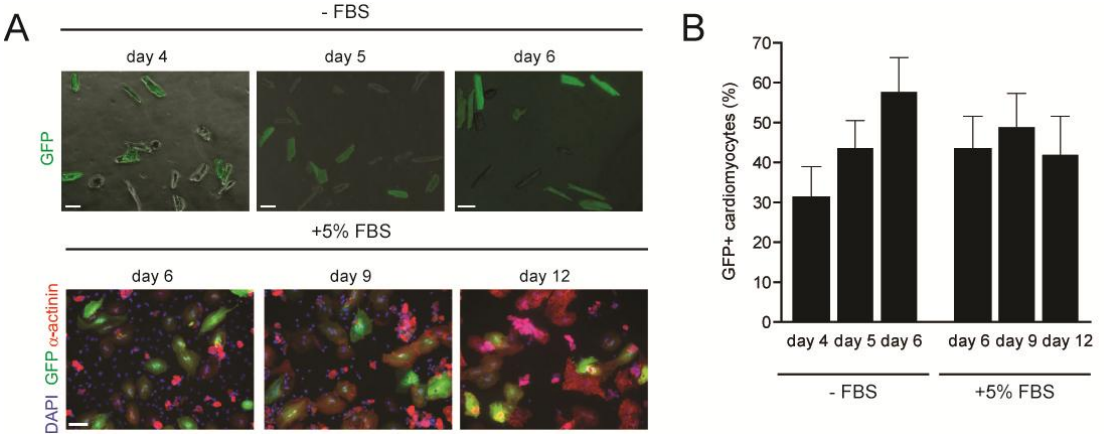


Figure 39. Transduction efficiency of adult cardiomyocytes by AAV serotype 6 vectors.

(**A**) Top panel: contrast phase representative images of adult rat cardiomyocytes cultured in absence of FBS at days 4, 5 and 6 after AAV6-EGFP transduction. Bottom panel: Representative immunofluorescence stainings of adult rat cardiomyocytes cultured in medium with 5% FBS at days 6, 9 and 12 after AAV6-EGFP transduction.

(**B**) Quantification of GFP+ adult cardiomyocytes after transduction with AAV6-EGFP vector in the absence or presence of FBS in the culture medium.

To investigate the effect of the Notch pathway stimulation on adult cardiomyocytes proliferation, we established a culture protocol following the same experimental conditions used for neonatal cells. In the presence of 5% of fetal bovine serum, adult cardiomyocytes undergo a dedifferentiation process, which leads to a radical morphological change in the adult cardiomyocytes, which lose their rod-like shape, but still are incapable of BrdU incorporation in the absence of stimuli (**Fig.40A**, panel a). Using these settings, the day after plating adult cardiomyocytes were transduced with either AAV6-N1ICD or AAV6-sJ1 and kept in culture for 9 days; 48 hours before fixation, BrdU was added to the medium. In these conditions, neither of the two vectors was able to stimulate BrdU incorporation (**Fig.40A**, panels c and e). When de-differentiated, adult cells were transduced with AAV6 encoding for miR-199a or miR-590 a BrdU pulse of 48 hours resulted in 5.7% and 16.2% of BrdU+ positive cardiomyocytes respectively (**Fig.40A**, panels g and i; **Fig.40B** for quantification), meaning that it is possible to re-activate proliferative pathway in adult cells, but not through direct stimulation of the Notch pathway. Adult cardiomyocytes transduced with miRNAs were also found positive for other cell cycle markers, like Ki67 and histone 3 serine 10 phosphorylation (**Fig.40C**).

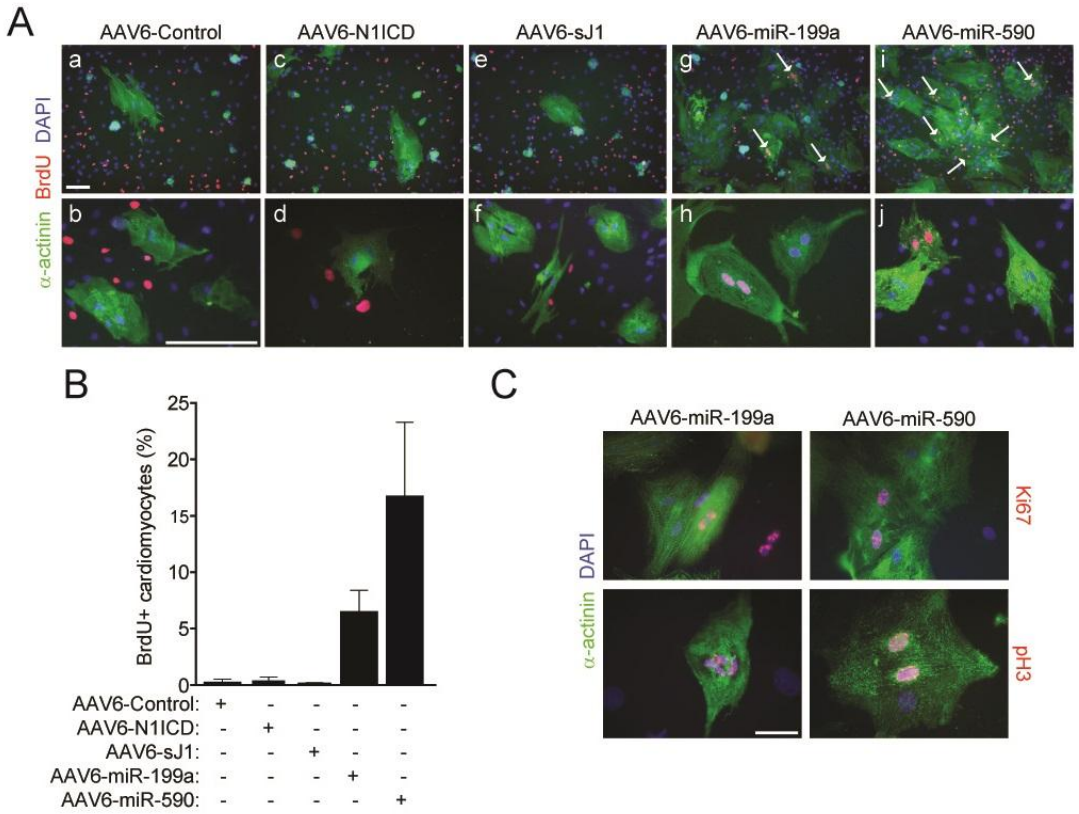


Figure 40. Notch1 pathway stimulation fails to induce adult cardiomyocyte re-entry into the cell cycle.

(**A**) Representative images of adult rat cardiomyocytes at day 9 of culture upon transduction with a control AAV6 (panel **a**), AAV6-N1ICD (**c**), AAV6-sJ1 (**e**), AAV6-miR-199a (**g**) or AAV6-miR-590 (**i**). Panels **b, d**, **f**, **h** and **j** show magnifications. White arrows point at cardiomyocyte nuclei positive for BrdU. Scale bar 100 µm.

(**B**) Quantification of adult cardiomyocyte proliferation after transduction with the indicated AAV6 vectors. Shown are the means±sem of at least three independent experiments.

(**C**) Representative images of adult rat cardiomyocytes at day 9 of culture upon transduction with a AAV6-miR-199a or AAV6-miR-590. Cardiomyocytes are stained in green with an anti-α-actinin antibody; nuclei are visualized in blue by DAPI. In the top panel red staining represents Ki67, in the bottom panel it represents histone 3 serine 10 phosphorylation (pH3). Scale bar 30 µm.

To further confirm that proliferation of adult cardiomyocytes does not involve the Notch1 signaling cascade, we evaluated the Notch target gene expression levels in adult cardiomyocytes transduced with either AAV6-miR-199a or AAV6-miR-590: none of the analyzed genes showed transcriptional upregulation, meaning that the proliferative program which is reactivated by selected miRNAs in the adult cells does not involve Notch signaling (**Fig.41**).

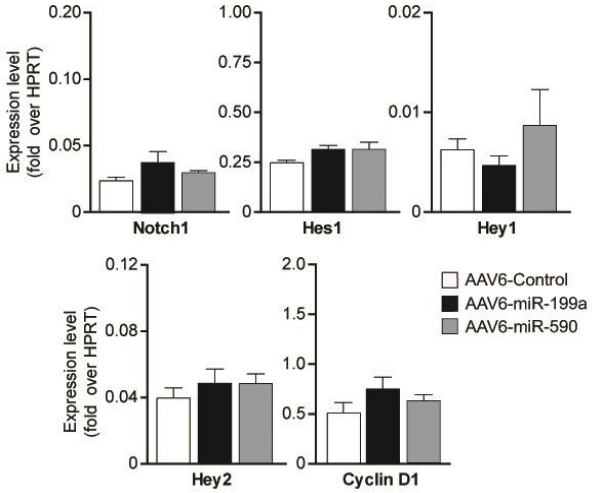


Figure 41. Notch target genes are not upregulated in proliferative adult cardiomyocytes.

Transcription levels of Notch1 and its indicated target genes in adult rat cardiomyocytes analyzed at day 9 after transduction with a control AAV6, AAV6-miR-199a or AAV6-miR-590. Data are expressed to cellular HPRT mRNA levels. Shown are the mean±sem of at least three independent experiments.

NOTCH TARGET GENE PROMOTERS ARE METHYLATED AT THE DNA LEVEL IN ADULT CARDIOMYOCYTES

To better understand the molecular cause for the lack of Notch stimulation in adult cells, we analyzed the DNA methylation pattern at Notch target gene promoters, as DNA methylation in the promoters is commonly associated with permanent transcriptional silencing [338]. For this purpose, we performed bisulfite sequencing analysis on genomic DNA extracted from neonatal cardiomyocyte kept in culture for 3 or 7 days, and from adult cardiomyocytes. Bisulfite sequencing analysis allows one to distinguish whether CpG dinucleotides are methylated or not in the DNA. Through bioinformatics analysis, we identified CpG-rich regions in the Notch target gene promoters. We performed bisulfite treatment on genomic DNA, followed by PCR in different areas of the CpG-rich regions spanning the transcriptional start site (TSS). Then we cloned and sequenced 8 different clones for each PCR amplified fragment.
Notch1 promoter displayed a strong increase of DNA methylation level paralleling the exit of cardiomyocytes from the cell cycle. At day 3, the extent of DNA methylation at the *Notch1* gene promoter was relatively low (2.5% of the CpG dinucleotides analyzed upstream the TSS and 1.8% downstream the TSS. Each row represents a single analyzed DNA clone; white and black circles show unmethylated and methylated CpG dinucleotides, respectively), markedly increased in day 7 cells (20.0% and 4.9%, respectively) and peaked in adult cardiomyocytes (36.2% and 12.9% respectively; **Fig.42A**). The same trend was also maintained in the *Hes1* gene, where, at day 3, 2.7% of the CpGs in the analyzed region upstream the TSS scored positive for methylation and 2.3% in the region downstream the TSS. These levels rose to 4.2% and 8.6% at day 7 and to 15.3 and 22.7%, respectively, in adult cardiomyocytes (**Fig.42B**). Analogous results were obtained for *Hey1* and *Hey2*, even if less pronounced, overall showing increased methylation in day 7 and adult cardiomyocytes compared to day 3 cells (**Fig.42C** and **42D** respectively). Of

potential interest, the *Cyclin D1* promoter showed a different methylation pattern, with scattered methylation also occurring in day 3 samples in the more upstream GC-rich region (2.5 kb upstream the TSS), not correlating with transcriptional silencing, while the CpG-rich regions close to the TSS (explored by primer set D) remained unmethylated also in the adult (**Fig.42E**). This result is consistent with the requirement for *Cyclin D1* re-expression when adult cardiomyocyte proliferation is induced by various stimuli, including miRNAs.

We also performed the same analysis of the TSS regions of *GAPDH*, a housekeeping gene, and *GFAP*, a glial gene silenced in cardiomyocytes, considering them as negative and positive controls for methylation analysis (**Fig.42F** and **42G** respectively).

	day 3	day 7	adult
Set A	2/80 (2.5%)	16/80 (20.0%)	29/80 (36.2%)
Set B	4/224 (1.8%)	11/224 (4.9%)	29/224 (12.9%)

	day 3	day 7	adult
Set A	2/72 (2.7%)	3/72 (4.2%)	11/72 (15.3%)
Set B	3/128 (2.3%)	11/128 (8.6%)	29/128 (22.7%)

	day 3	day 7	adult
Set A	1/152 (0.6%)	16/152 (10.5%)	10/152 (6.6%)
Set B	0/120 (0%)	2/120 (1.7%)	7/120 (5.8%)

	day 3	day 7	adult
Set A	0/80 (0%)	1/80 (1.25%)	4/80 (5%)

	day 3	day 7	adult
Set A	11/232 (4.7%)	23/232 (9.9%)	23/232 (9.9%)
Set B	4/80 (5%)	13/80 (16.2%)	16/80 (20%)
Set C	7/152 (4.6%)	15/152 (9.9%)	19/152 (12.5%)
Set D	6/152 (4.0%)	2/152 (1.3%)	5/152 (3.3%)

	day 3	day 7	adult
Set A	4/176 (2.3%)	5/176 (2.8%)	3/176 (1.7%)
Set B	0/96 (0%)	0/96 (0%)	1/96 (1.0%)

	day 3	day 7	adult
Set A	139/176 (77.8%)	141/176 (80.1%)	160/176 (90.9%)

Figure 42. *Notch1* and *Hes1* promoters in non proliferative cardiomyocytes show markedly increased levels of DNA methylation.

(**A**-**G**) Results of bisulfite sequencing data from DNA obtained from day 3, day 7 and adult rat cardiomyocytes using primer pairs encompassing the indicated promoter regions of the *Notch1* (**A**), *Hes1* (**B**), *Hey1* (**C**), *Hey2* (**D**), *Cyclin D1* (**E**), *GAPDH* (**F**) and *GFAP* (**G**) genes (the last two serving as negative and positive controls for methylation respectively).

DNA methylation is widely associated to transcriptional repression, but recently few cases of opposite behavior have been identified [430]; we therefore needed to confirm that in the genes of our interest DNA methylation spanning the TSS effectively corresponds to transcriptional silencing. We performed an in vitro methylation assay, in which different constructs containing *Notch1*, *Hes1*, *Hey1* and *Hey2* promoters were cloned upstream the Luciferase reporter gene; the empty vector pGL3 was used as a control. These plasmids were in vitro methylated using SssI CpG methyltransferase and the methyl donor S-adenosyl-methionine (SAM). They were subsequently transfected in neonatal rat cardiomyocytes and Luciferase activity was analyzed at days 3 or 7 of culture (**Fig.43A** and **43B** respectively). We observed that DNA methylation markedly decreased Luciferase activity, further supporting the evidence that DNA methylation correlates with repressed transcription at Notch target gene promoters.

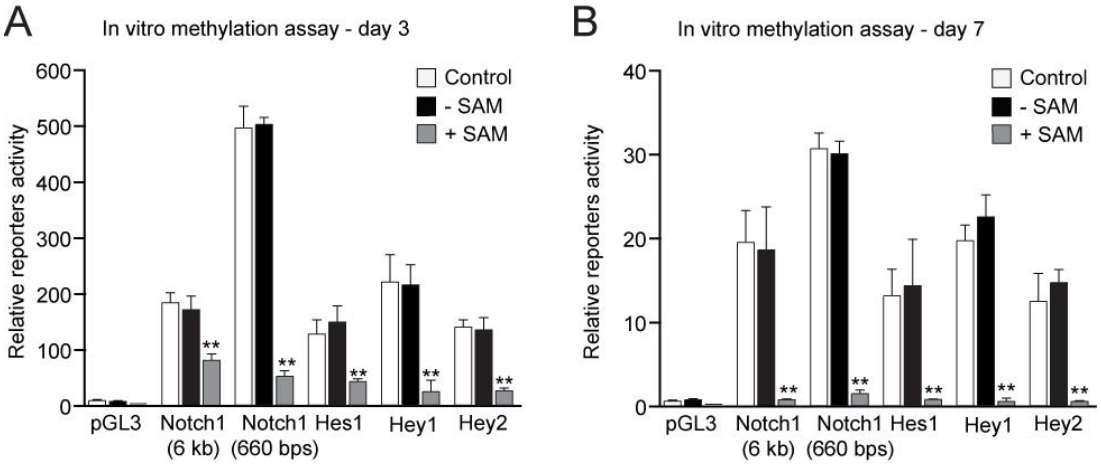


Figure 43. Effect of promoter methylation in Luciferase reporter assays.
(**A** and **B**) Rat neonatal cardiomyocytes were transiently transfected with SssI-methylated (grey bars) or unmethylated (white bars) plasmids containing either *Notch1* full length or the *Notch1* core region promoter, *Hes1*, *Hey1*, *Hey2* driving expression of Firefly Luciferase. As a control of the methylation reaction, each plasmid DNA vector was also treated with equal amounts of methylating enzyme, but without the methyl group donor SAM (black bars). Luciferase activities were normalized for transfection efficiency by cotransfection of an unmethylated Renilla construct. The empty vector (pGL3) was used as negative control.

Values are the mean ± sem obtained from three independent experiments. **: $P< 0.01$. Reporter gene expression was analyzed at days 3 (**A**) and 7 (**B**).

In vitro DNA methylation efficiency was assessed comparing DNA sensitivity to the MspI/HpaII restriction enzyme isoschizomers: in the presence of the methyl donor SAM, the methylation-sensitive enzyme HpaII was unable to digest the plasmid, confirming the efficient methylation of the constructs (**Fig.44**).

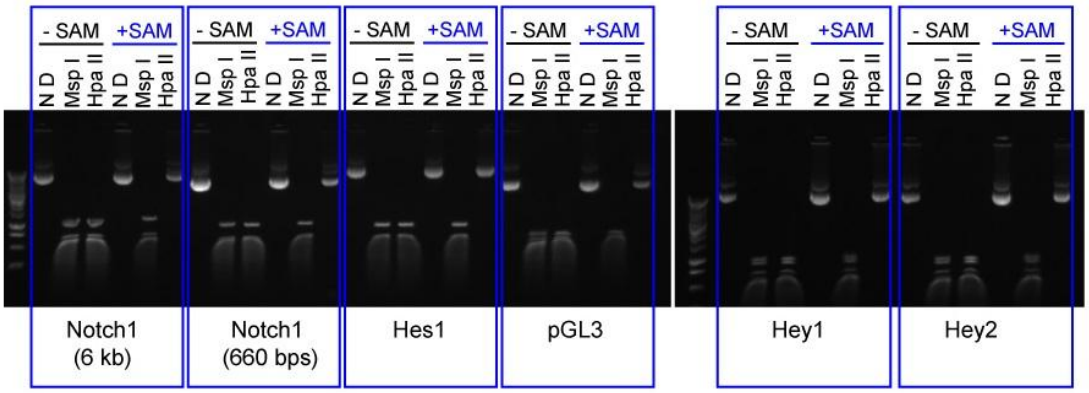
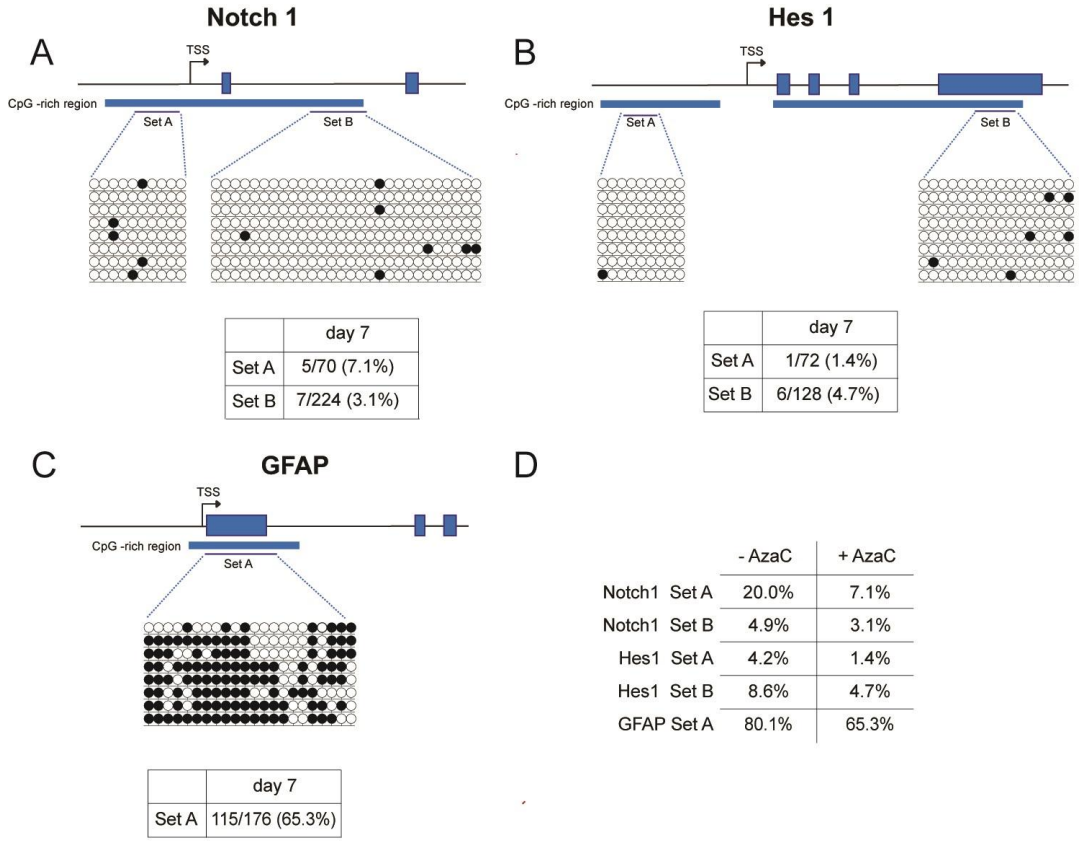


Figure 44. Control of the in vitro methylation efficiency.

To further demonstrate the role of DNA methylation as an important regulator of Notch pathway silencing, we cultured neonatal cardiomyocytes in the presence of 5-Aza-2´deoxycytidine (AzaC), a nucleotide analog that prevents DNA methylation. AzaC treatment is effective as DNA methylation inhibitor during the establishment of the methylation pattern, while it cannot revert DNA methylation when it is already present. Therefore, we performed the experiment in neonatal cardiomyocytes, in order to prevent DNA methylation deposition, and not in adult cells, in which DNA methylation is already present, since AzaC treatment would have been ineffective. We treated cultured cardiomyocytes for 48 hours between days 1 and 3 of culture by adding 5 μM AzaC in the medium. We first wanted to analyze whether this treatment affected the DNA methylation pattern. For this purpose, we performed bisulfite sequencing analysis of genomic DNA of day 7 cardiomyocytes treated with

AzaC. We analyzed the methylation pattern of *Notch1*, *Hes1* and *GFAP* (**Fig.45 A**, **B** and **C** respectively), and we detected a strong reduction at the DNA methylation level compared to the control (summarized in **Fig.45D**).



	day 7
Set A	5/70 (7.1%)
Set B	7/224 (3.1%)

	day 7
Set A	1/72 (1.4%)
Set B	6/128 (4.7%)

	day 7
Set A	115/176 (65.3%)

	- AzaC	+ AzaC
Notch1 Set A	20.0%	7.1%
Notch1 Set B	4.9%	3.1%
Hes1 Set A	4.2%	1.4%
Hes1 Set B	8.6%	4.7%
GFAP Set A	80.1%	65.3%

Figure 45. AzaC treatment decreases DNA methylation in the analyzed genes.
(A-C) Results of bisulfite sequencing data from DNA obtained from day 7 cardiomyocytes treated, between day 1 and day 3, with 5 μM AzaC. Eight different clones are shown for *Notch1* (**A**), *Hes1* (**B**) and *GFAP* (**C**).
(**D**) Summary table showing differences in DNA methylation found in day 7 cardiomyocytes comparing untreated cells (data shown in Fig.20) and cells treated with AzaC.

We then analyzed the effect of AzaC treatment on cardiomyocyte proliferation at day 7. By itself, AzaC supplementation to the culture medium is able to strongly stimulate BrdU incorporation in cardiomyocytes (which reaches 15% of BrdU+, α-actinin+ cells), consistent with the fact that inhibition of DNA methylation blocks the

silencing of many pathways controlling proliferation activity in the fetal life, later downregulated in the postnatal life. When we transduced AzaC-treated cardiomyocytes with AAV6-N1ICD, we detected a strong synergistic effect between Notch stimulation and AzaC treatment, with 25.9% of cardiomyocytes which scored positive for BrdU at day 7. The most relevant result, however, was the effect of AzaC in creating a favorable chromatin environment for the NICD driven transcriptional regulation of cardiomyocyte proliferation. We transduced cultured cardiomyocytes at day 4 with AAV6-N1ICD, and in this case the N1ICD transgene was less effective in triggering cardiomyocyte proliferation compared to cells transduced at day 0 (10.9% vs. 17.2%). In the presence of AzaC, even in case of transduction at day 4, cardiomyocytes reached 26.2% of BrdU positive cells, consistent with the conclusion that impeding the establishment of de novo methylation favors the effect of N1ICD (**Fig.46**). Therefore, we can conclude that exit from the cell cycle of cardiomyocytes correlates with the permanent repression of Notch1 target genes, therefore providing a molecular explanation for the inefficacy of the treatments aimed at reactivation of Notch pathway by gene transfer as a mean to induce cardiac regeneration in vivo.

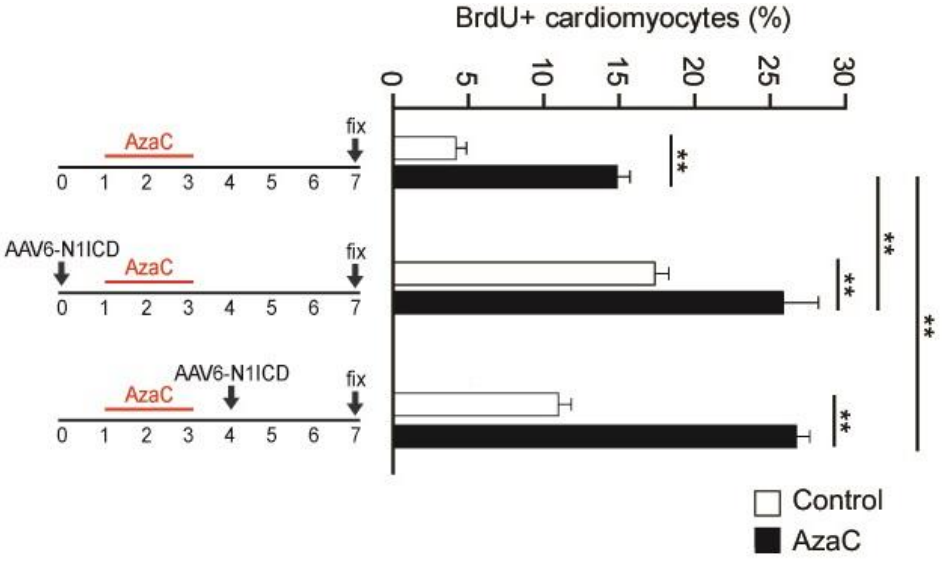


Figure 46. Inhibition of DNA methylation synergizes with Notch activation in stimulating cardiomyocyte proliferation.

Number of proliferating cardiomyocytes analyzed at day 7 in cultures transduced at day 0 or day 4 with AAV6-N1ICD and treated with AzaC at days 1-3 when indicated. Shown are the means±sem of at least three independent experiments. **: $P<0.01$. "Control" (white bar) in the first group of two from top corresponds to untreated cells; "Control" in the lower two groups corresponds to cells transduced with AAV6-N1ICD but not with AzaC.

DISCUSSION

There is a pressing need to develop novel therapeutic approaches aimed at generating new contractile tissue in patients with myocardial damage. In mammals, the heart tries to react to the loss of cardiomyocytes with a partial regenerative attempt [25-26], but the prevailing mechanism in response to myocardial damage is scarring. Many approaches have been used to achieve heart regeneration via the induction of cardiomyocyte proliferation [32-37]. My thesis is in this line of research, studying a possible regenerative role of the Notch pathway in the heart.

The Notch pathway is a widely known regulator of proliferation during many developmental processes and it plays a crucial role during cardiogenesis [192]. It is well established that cardiomyocytes are actively proliferating in the fetal and early neonatal life, but lose their proliferative potential shortly after birth. Previous results from our lab [196] and others [203] reported an important role of Notch signaling in the control of neonatal cardiomyocyte proliferation. In details, we noticed that at birth a relevant fraction of immature, still proliferating cardiomyocytes are positive for Notch1 expression, while this population is lost over time, since in the adult the fraction of proliferative cardiomyocytes is significantly lower. In a model of rat cardiomyocyte culture, we can recapitulate the exit of cardiomyocytes from the cell cycle, paralleled by the loss of Notch signaling. In vitro, the sustained activation of this pathway through a gene transfer approach using an AAV coding for the active form of Notch1 (N1ICD) or through the stimulation with the soluble form of the ligand Jagged1 (sJ1), is able to expand the fraction of immature ventricular cardiomyocytes still capable of proliferation. Accordingly, the block of Notch1 signaling significantly reduces cardiomyocyte proliferation potential [196]. In line with these evidences, exogenous administration of Notch2-ICD was demonstrated to induce cardiomyocyte cell cycle re-entry. N2ICD not only induces the expression of

the cell cycle regulator Cyclin D1 but also triggers Cyclin D1 import in the nucleus, therefore confirming the relevance of Notch pathway in cardiomyocyte proliferation control [203].

In Zebrafish, upon the resection of up to 20% of the ventricle, cardiac regeneration is efficiently achieved through adult cardiomyocyte proliferation. This process is controlled by the Notch pathway, which is strongly activated both in the endocardium and in the epicardium of amputated Zebrafish hearts during the healing process. The block of the Notch signaling profoundly impairs the regenerative capacity of the Zebrafish heart, decreasing the fraction of proliferative cardiomyocytes and inhibiting the atrial to ventricular transdifferentiation process. Interestingly these findings demonstrate the existence of a specific regenerative pathway, different from the embryonic heart development program [96, 99-101].

Given the above reported evidences, we wondered whether the Notch pathway could drive cardiomyocyte proliferation in adult rodents as well as it happens in the Zebrafish heart. The rationale behind this approach was that the genetic program downstream its activation could eventually lead to myocardial regeneration after damage, therefore supporting the exploiting of Notch1 as a therapeutic tool for cardiac regeneration.

The in vitro studies involved a comparative analysis of the Notch pathway in neonatal and adult cardiomyocytes. Our culture model of neonatal cardiomyocytes reproduces in vitro the withdrawal of myocytes from the cell cycle, which happens in vivo during the first week after birth: we considered 3 days-old cardiomyocytes as "proliferative" and 7 days-old cardiomyocytes as "non proliferative". In order to have a more physiological model for adult cells, we optimized the Langendorff perfusion system to purify and culture cardiomyocytes from adult rats.

We show that Notch pathway sustains active cardiomyocyte proliferation and that cardiomyocyte loss of proliferation capacity together with their exit from the cell cycle coincide with the epigenetic suppression at Notch target genes, which

correlates with the decreased transcriptional level of Notch target genes. In this context we have analyzed the genes described to be downstream of Notch signaling during heart development (as basic helix-loop-helix transcription factors *Hes1*, *Hey1* and *Hey2*) and *Cyclin D1*, important regulator of cell cycle controlled by Notch in cardiomyocytes [203, 431] and *Notch1* itself [426].

The regulation of Notch pathway involves a variety of epigenetic modifications occurring at the target gene promoters [112]: the presence of NICD contribute to the formation of a complex promoting active chromatin transcription, while after the degradation of NICD, in order to switch off the transcription of the target genes, a transcriptional repressor complex is recruited. We further characterized the epigenetic modifications occurring in cardiomyocytes at Notch target gene promoters: we observed that, in neonatal cardiomyocytes, these chromatin regions are in an open and transcriptionally permissive conformation, supporting the correlation between the high levels of transcription with the proliferative capacity of the cells. The analyzed promoters are characterized by the presence of broad marks of active transcription, as pan-acetylation of histone 3 and H4K3 trimethylation [432-433], consistent with the reported data, stating the presence in the NICD activator complex of different HATs [112, 116] and the association of H3K4 trimethylation with NICD-driven active transcription [365, 369].

Interestingly, one week after birth and in adult cells, the presence of the same active chromatin marks is strongly decreased, while marks of repressive chromatin associated to Polycomb mediated silencing [268, 272], as H3K27me3 and the promoter occupancy by the methylating enzyme Ezh2 [266, 274, 282] are enriched. These data support the idea that at, Notch responsive genes, the chromatin environment passes from a transcriptionally active to an inactive state parallel to cardiomyocyte terminal withdrawal from the cell cycle and that this repressive environment is established by the Polycomb Group proteins.

Polycomb-mediated silencing of Notch pathway is also reported in other models: during development in Drosophila, where PRC1 binds to multiple components of Notch signaling pathway to control proliferation [371], in dystrophic skeletal muscle, where TNFα suppresses Notch1 promoting the recruitment of Ezh2 to the Notch-responsive promoters [377] and in B cell acute lymphoblastic leukemia, where Notch and its target genes are less expressed due to epigenetic regulation at the promoters, involving decreased H3K4 methylation and increased H3K9 and H3K27 methylation [378]. Moreover, a genome wide study aimed to analyze the location of H3K27 trimethylation in embryonic fibroblasts, identified the Notch signaling as one of the pathway regulated by PcG-mediated silencing [281]. Interestingly, PRC2 complex has been reported to interact with H3K4 demethylases [434]. In the developing myocardium, endothelial deletion of Jarid2, a member of the Jumonji family of proteins which associates with the PcG complex to modulate its function [435-436], derepresses endocardial Notch1 expression [373]. In addition, deletion of Ezh2 in cardiac progenitors is known to cause postnatal myocardial pathology [428]. The results shown in this Thesis appears to be fully consistent with these findings.

After assessing the repressive chromatin environment at Notch target genes in fully differentiated cardiomyocytes, we wondered whether it was possible to revert the silencing using a gene transfer approach, exploiting the Adeno Associated viral (AAV) vector technology.

AAV vectors are excellent tool to achieve long lasting transgene expression in postmitotic organs in vivo, being capsid 9 the most efficient serotype to achieve high transduction levels in the heart in vivo [407] as well as a very interesting tool to efficiently transduce cardiomyocytes in vitro, achieved using serotype 6 capsid [437]. Therefore we exploited AAV technology to force Notch pathway activation. We designed two AAV vectors, able to activate the Notch pathway through different strategies: the AAV-N1ICD codes for the intracellular domain of Notch1, which can directly migrate into the nucleus, interact with RBP-Jk and recruit the transcription

activator complex, turning on the transcription of Notch target genes irrespective of the endogenous level of Notch1 expression in the transduced cells. The vector encoding for the soluble form of Jagged1 instead targets a different cell population, since it activates signaling only in the cells that express endogenous Notch1 receptor, and in which Notch activation can create a feedback loop stimulating its own expression. Moreover, in order to activate signaling, the Jagged1 transgene needs to reach a minimum concentration in the medium, necessary for the multimerization step required for soluble ligand-mediated activation of Notch signaling [438]. This conceptual difference between the two vectors explains their different strength and kinetics.

In neonatal cardiomyocytes, the establishment of repressive chromatin environment at Notch target genes was efficiently counteracted by the exogenous activation of Notch pathway achieved by AAV-mediated gene transfer using both the NICD and soluble Jagged1 transgenes. Transduction correlated with increased transcriptional activation of Notch target genes after 7 days of culture, with elevated and prolonged proliferative capacity of cardiomyocytes in culture and with the maintenance of a transcriptionally competent chromatin environment. Moreover, the synergy in sustaining proliferation between Notch pathway stimulation by AAV and Ezh2 inhibition, which prevents the deposition of Polycomb related repressive marks, further strengthened the role of Polycomb-mediated silencing in the establishment of a repressor environment at Notch target gene promoters.

To our disappointment, the AAV-mediated reactivation of the Notch pathway was ineffective in vivo in a model of myocardial infarction, where we expected that Notch activation, similar to its effect in neonatal cardiomyocytes, would have stimulated cardiomyocytes to proliferate, therefore sustaining a regenerative response in the infarcted heart. On the contrary of our expectation, no regenerative response, and hence no major beneficial effect, was apparent in our vivo experiment. NICD-transduced mice had no functional better outcome compared to controls, while sJ1-

transduced animals showed a trend, which was not statistically significant yet, towards improved function. We speculate that this marginal effect might be the consequence of the action of Jagged1, which is expressed and secreted by the transduced cardiomyocytes, on other cell types than cardiomyocytes, for example stimulating angiogenesis or modulating the immune system response.

Interestingly, adult cardiomyocytes are not replicative cells, but they can be induced to reenter in the cell cycle in response to a variety of stimuli, including Periostin [36], Neuregulin [37], FGF1 together with a p38 inhibitor [34], miR-590 and miR-199a [39], which have all been shown capable to stimulate DNA synthesis, karyokinesis and cytokinesis; the same stimuli, then, also provided benefit after myocardial infarction, resulting in improved cardiac functionality and reduced infarct size. Therefore, the lack of regenerative response driven by Notch is not due to an intrinsic inability of adult cardiomyocytes to proliferate, but to the inefficiency of Notch to activate the specific genetic program underlying the proliferative capacity in adult cells.

A molecular explanation for the different behavior of neonatal and adult cells in respect to Notch pathway stimulation resides in the different methylation pattern found at the DNA level in the Notch target genes. We detected that the DNA methylation at these promoters was increased over time, paralleling cardiomyocyte exit from the cell cycle and explaining the lack of action in the adult, since DNA methylation is commonly associated with gene repression [338]. Interestingly, the *Cyclin D1* promoter behaved differently at the DNA methylation level, showing no clear methylation enrichment from neonatal to adult cardiomyocytes. This result is in agreement with the requirement for Cyclin D1 re-expression when proliferation of adult cardiomyocytes is triggered by other stimuli.

The relationship between Polycomb-mediated gene repression and DNA methylation is still poorly understood. In many contexts these two occurrences are probably mutually exclusive; however in embryonic stem cells, genes marked by H3K27 methylation are more likely to acquire DNA methylation [325-326], suggesting a

potential cooperation between the two mechanisms. During development, the silencing process is initiated by Polycomb complexes and, subsequently, long term silencing is achieved via DNA methylation, also due to the Ezh2-mediated recruitment of Dnmts [327]. Interestingly, this silencing step cascade has been reported at the *Notch* promoter in skeletal muscle cells [377] and in B-cell acute leukemia cells [378], where Polycomb mediated silencing induces DNA methylation.

The literature is rich of reports showing a beneficial effect of Notch signaling activation in a variety of models of heart damage. Notch signaling has been reported to exert regenerative effect through activation of an epicardial derived cell population, which could decrease fibrosis, stabilize the arterial endothelium and control angiogenesis [200]. Notch can act on a mesenchymal stromal cell population, where it reduces myofibroblast proliferation, while it stimulates cardiac precursor cell proliferation [199]; it can recruit bone marrow derived cells in the context of heart infarction, where they stimulate neovascularization and infarct size reduction [439]; it stimulates progenitor cell population expansion and commitment toward mature cardiomyocytes [194, 202]. Remarkably, all these beneficial effects appear to act through the stimulation of presumed or demonstrated stem cell populations or through the stimulation of the intrinsic capacity of still not fully differentiated cardiomyocytes to proliferate. In contrast, our strategy, which was based on the stimulation of adult cardiomyocyte proliferation, turned out to be ineffectual, but this is not in contrast with previously described data. Notch pathway stimulation could still remain a possible tool to expand precursor cell or immature cardiomyocyte populations, which could then contribute to heart regeneration; moreover, the marginal improvement in cardiac function observed in vivo in the animals transduced with AAVsJ1, which can be attribute to paracrine effect, supports a possible beneficial effect of Notch1 stimulation in myocardial infarction on other cell types than cardiomyocytes. Published work has also reported that the conditional overexpression of Notch1 in cardiomyocytes after myocardial infarction in an α-myosin heavy chain-mER-Cre-mER transgenic mice showed a positive effect,

which was mainly attributed to the preservation of cardiomyocyte viability and the stimulation of angiogenesis, with no evidence of a major regenerative effect [197]. This conclusion appears to be fully consistent with our results.

In conclusion, contrary to other conditions such as brain ischemia [154] and skeletal muscle injury [440] in which reactivation of Notch signaling promotes tissue repair, attempts at inducing cardiac regeneration after myocardial infarction by the reactivation of the Notch pathway in cardiomyocytes by gene transfer appear to be ineffective. This observation contrasts with the assumption that tissue regeneration in adult organisms has to recapitulate the events occurring during development. A parallel situation also exists for other organs. For instance, the satellite cell transcription factor Pax7 is required for skeletal muscle regeneration in neonatal mice, but is dispensable for regeneration during juvenile and adult stages [441]. In both cardiac and skeletal muscle, therefore, it appears that a distinction exists between the molecular mechanisms driving development and those responsible for further maintenance, expansion and repair of the differentiated tissues.

The therapeutic exploitation of proliferation mechanisms in adult tissues is still a highly sought target for future investigation.

MATERIALS AND METHODS

Animals

Animal care and treatment were conducted in conformity with institutional guidelines in compliance with national and international laws and policies (European Economic Community Council Directive 86/609, OJL 358, December 12, 1987). Wistar rats and CD1 mice were purchased from Charles River Laboratories Italia Srl and maintained under controlled environmental conditions.

Culture of neonatal rat cardiomyocytes

Neonatal rat ventricular cardiomyocytes were extracted from day 0 or day 1 newborn Wistar rats, as described previously [196], with minor modifications. Briefly, ventricles were separated from atria and great vessels and cut in smaller pieces. The dissociation was performed in CBFHH (calcium and bicarbonate-free Hanks with Hepes) buffer containing 1.75 mg/ml of trypsin (BD Difco) and 10 µg/ml DNAse II (Sigma). Digestions were performed on a stirrer in eight- to ten-10 minutes steps; after each digestion, the supernatant was collected and trypsin was inactivated with Fetal Bovine Serum (FBS, Life Technologies). The collected supernatant was centrifuged and the cell pellet was resuspended in the medium DMEM (Dulbecco's modified Eagle medium, Life Technologies) 4.5 g/l glucose supplemented with 5% FBS, 20 mg/ml vitamin B12 (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma). The cells were filtered through a cell strainer (40 µm, BD Falcon) and then pre-plated on plastic 10 cm dishes for 2 hr at 37° in 5% CO_2. After the pre-plating step, the cardiomyocyte-containing medium was collected, the cells were counted and then plated at the appropriate density on Primaria plates (BD Falcon), or on glass slides pre-coated with 0.2% gelatin in PBS. The culture medium was replaced with fresh one the day after plating.

When indicated, the Ezh2 inhibitor GSK126 [429] (BioVision; 1 μM) was added to the medium for 2 or 4 days. When indicated, 5-Aza-2-deoxycytidine (AzaC; Sigma; 5 μM) was added to the culture medium for the first 2 days of culture, between day 1 and day 3, and then removed. When indicated, the γ-secretase inhibitor DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester, Sigma, 10 μM for 8 hours) was added. In all the cases, DMSO was used as a control.

Culture of adult rat cardiomyocytes

Adult ventricular cardiomyocytes from 2-months-old adult female Wistar rats were extracted with Langendorff perfusion system. Animals were anesthetized with zoletil and xylazine and heparinized with 2 ml of heparin (Sigma) 1000U/ml. The heart was cannulated through the aorta and perfused with a perfusion buffer composed of NaCl 120.4 mM, KCl 14.7 mM, KH_2PO_4 0.6 mM, Na_2HPO_4 0.6 mM, $MgSO_4$-$7H_20$ 1.2 mM, $NaHCO_3$ 4.6 mM, Na-Hepes 10 mM, taurine 30 mM, 2,3-butanedione monoxime (BDM) 10 mM, glucose 5.5mM (all from Sigma) in H_2O pH 7.2. After 5 min, 1 mg/ml Liberase TM (Roche) was added to the perfusion buffer for 10-12 min. The heart was detached from the apparatus; atria and great vessels were removed and the ventricles were cut into smaller pieces. Mechanical digestion was performed pipetting up and down the tissue fragments in wash medium (50:50 perfusion buffer: DMEM 1g/l glucose). The collected cell suspension was filtered (100 μm cell strainer, BD Falcon) and centrifuged at low speed (30 g) 3 min at room temperature. The cell pellet was resuspended in wash buffer and then added to the BSA gradient prepared with 0.645 g BSA (Sigma) in DMEM 1g/l glucose. The separation of cardiomyocytes from other cell types lasted 15 min at room temperature. Then the cardiomyocyte pellet was resuspended in ACCT medium, composed of 2 g/l BSA, 2 mM L-carnitine, 5 mM creatine, 5 mM taurine, 1 mM BDM (all from Sigma), 100 U/ml of penicillin and 100 μg/ml of streptomycin in DMEM 1g/l glucose. Cells were plated on Primaria plates coated with Laminin (Sigma) and kept in culture at 37° in 5% CO_2. Two hr after plating, the medium was replaced with

fresh ACCT medium to remove cardiomyocytes not attached to the plate. The medium was changed 24 hr later with fresh ACCT medium or with DMEM 4.5 g/l glucose supplemented with 5% FBS and Vitamin B12.

RNA isolation and quantitative real-time PCR

Total mRNA was purified either from cultured neonatal cardiomyocytes at day 3 and 7 of culture, or from adult cardiomyocytes 3 or 9 days after plating or from total heart homogenates. Extracted mRNA (1 µg) was reverse-transcribed using MLV-RT (Invitrogen) with random hexamers (10 µM) in a 20 µl reaction, following the manufacturer's instruction. mRNA levels for Notch1, Hes1, Hey1, Hey2, Cyclin D1 and HPRT genes (primer sequences listed in Primer Table I) were quantified by qRT-PCR and GoTaq qPCR Mater Mix (Promega). The real-time qPCR program was performed with a melting curve dissociation protocol (from 60°C to 95°C), according to manufacturer's instruction. The final dilution of the primers in the reaction was 900 nm; for each primer set, optimal conditions were established and efficiency of the amplification was calculated.

Analysis of protein expression

For the transgene expression, heart samples were collected at different time points after myocardial infarction, homogenized in 1 ml of RIPA Buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% deoxycholate), supplemented with 90 µg/ml PMSF, 1 mM Na_3VO_4 (all from Sigma) and protease inhibitors (Roche), using Magna Lyser (Roche). After sonication and pre-clearing, protein lysate concentration was determined by Bradford Assay (Biorad). Equal amounts of proteins were resolved on 6% SDS-PAGE minigels and transferred to nitrocellulose membranes (GE Healthcare). Immunoblots were blocked in 5% skim milk in TBST (50 mM Tris-HCl pH 7.4, 200 mM NaCl, and 0.1% Tween 20). Membranes were incubated with primary antibodies overnight and washed in TBST. Secondary antibodies were diluted in blocking buffer and incubated with the

membranes for 45 min at room temperature. Proteins were detected with the ECL detection kit (GE Health Care Bio-Sciences).

For the analysis of H3K27 methylation, the same procedure was performed, but proteins were resolved on 15% if SDS-PAGE minigels. Membrane stripping was performed in stripping buffer (100mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) at 56°C for 15 min, followed by extensive washing in TBST.

To detect the in vivo physical interaction between N1ICD transgene and RBP-Jk, $1x10^6$ neonatal rat cardiomyocytes, transduced with AAV6-N1ICD, were lysed in IPLS buffer (50 mM Tris-HCl pH 7.5, 120 mM NaCl, 0.5 mM EDTA and 0.5% Nonidet P-40) supplemented with 90 µg/ml PMSF (Sigma) and protease inhibitors (Roche). After sonication and pre-clearing, protein lysate concentration was determined by Bradford Assay (Biorad). N1ICD was immunoprecipitated from total cell lysates with 1 mg/ml of monoclonal 9B11 Myc-tag antibody for 2 hr at 4°C with gentle rotation, followed by incubation with protein A/G agarose beads (Santa Cruz) for additional 2 hr. Immunoprecipitates were resolved on 10% SDS-PAGE minigels and transferred to nitrocellulose membranes (GE Healthcare). Immunoblots were blocked in 2% BSA (Roche) in TBST. Membranes were incubated with RBP-Jk primary antibody overnight and washed in TBST. Secondary antibodies were diluted in blocking buffer and incubated with the membranes for 45 min at room temperature. Proteins were detected with the ECL detection kit (GE Health Care Bio-Sciences).

The following antibodies were used for Western Blot: anti-Myc 9B11 (2279, 1:1000, Cell Signaling), anti-Tubulin (T5168, 1:10000, Sigma), anti-H3K27me3 (ab6002, 1:1000, Abcam), anti-Histone-H3 (06-755, 1:1000, Millipore), anti RBP-Jk (AB 2284, 1:1000, Millipore). The following antibody were used as secondary: goat anti-mouse conjugated to HRP (P0447, Dako-Cytomation), goat anti-rabbit conjugated to HRP (31460, Thermo Scientific), protein A conjugated to HRP (18-160, Millipore).

Luciferase assays

Neonatal cardiomyocytes were seeded onto 96-well primary cells culture plates ($1x10^4$ cells per well) and co-transfected after either 2 or 6 days after isolation with 0.5 µg of either pHes1 (kindly provided by R. Kageyama, Kyoto University, Japan) or 4xRBP-Jk-Luc (provided by S.D. Hayward, John Hopkins University School of Medicine, Baltimore, MD) reporter plasmids and 0.05 µg pRL-Renilla (which was used as a control) using Lipofectamine 2000 transfection reagent (Invitrogen). 24 hr after transfection, cells were harvested and both Firefly and Renilla Luciferase activities were assayed with the Dual-luciferase reporter assay kit (Promega). The value of Firefly Luciferase activity was corrected for the transfection efficiency by using the Renilla Luciferase activity in each sample.

Promoter methylation assays

A series of plasmids were generated, in which the promoter regions of the following genes were cloned upstream of the Firefly Luciferase gene: *Hes1* [442] (kindly provided by R. Kageyama, Kyoto University, Japan), *Hey1* [443] (kindly provided by Manfred Gessler, Theodor-Boveri-Institut fuer Biowissenschaften, Wuerzburg, Germany), *Hey2* [118] (kindly provided by Stefano Zanotti, Saint Francis Hospital and Medical Center, Hartford, CT) and *Notch1* [444] (kindly provided by Warren S. Pear, University of Pennsylvania, Philadelphia). Plasmid pGL3 contained an empty polylinker (Promega). Each reporter construct (10 µg) was methylated in vitro using 20 U of SssI methylase (New England Biolabs) in the presence of S-adenosyl-methionine (160 µM; New England Biolabs) at 37°C for 4 hr, with subsequent inactivation of the enzyme at 65°C for 20 min. Mock-methylation reactions were performed using the same conditions, but omitting SAM. Complete methylation was ascertained by digesting the methylated DNA with an excess (20 U/mg) of restriction enzymes HpaII or MspI. All the constructs were purified using Wizard DNA purification columns (Promega).

Neonatal cardiomyocytes were seeded onto 96-wells primary cells culture plates ($1x10^4$ cells per well) and co-transfected at either day 2 or 6 with 0.5 µg methylated or mock-methylated constructs and 0.05 µg pRL-Renilla (which was used for standardization) using Lipofectamine 2000 transfection reagent (Invitrogen). 24 hr after transfection, cells were harvested and both Firefly and Renilla Luciferase activities were assayed with the Dual-luciferase reporter assay kit (Promega). The value of Firefly Luciferase activity was corrected for the transfection efficiency by using the Renilla Luciferase activity in each sample.

Viral genome quantification through competitive PCR

DNA was extracted from frozen hearts of transduced animals collected at different time points with Blood and Tissue Kit (Qiagen). Competitive PCR was performed as previously described [445], using a multicompetitor plasmid carrying a deleted form of the cellular gene β-globin flanked by CMV primer sequences to quantify total genomic DNA as a reference and AAV vector DNA, respectively [446]. Fixed amounts of sample DNA were mixed with scalar amounts of the multicompetitor DNA and PCR was performed with the 2 primer sets. The PCR reaction was run on 8% polyacrylamide gel and stained with ethidium bromide; the different bands corresponding to competitor-, AAV- or the β-globin-DNA were quantified. The primers used for the competitor and for the PCR amplification are listed in Online Table II.

Chromatin immunoprecipitation (ChIP)

The protocol for immunoprecipitation of cardiomyocyte chromatin was adapted from that used in ref. [447]. Day 3, day 7 and adult cardiomyocytes (approximately $10x10^6$ cells for each time point) were cross-linked with 11% formaldehyde for 10 min at room temperature, followed by termination of the reaction with 125 mM glycine on ice. The cell pellet was washed twice in PBS, lysed in 2% NP-40 buffer (10 mM TrisHCl pH7.4, 10 mM NaCl, 3mM $MgCl_2$, 1 mM PMSF and protease

inhibitors) to obtain purified nuclei, followed by dounce homogenization in the same buffer. Lysis of the nuclei was performed using the same buffer containing 4% NP-40 and left at 37°C for 15 min, after which micrococcal nuclease (120 U, Roche) was added. The reaction was stopped after 15 min with 3 mM EGTA. DNA was additionally sheared by sonication to an average size of DNA fragments below 0.5 kb. Extracts were pre-cleared by 2 rounds of incubation with IgGs and agarose beads, followed by centrifugation at 3500 rpm for 5-10 min. The lysate (400 μl) was then incubated with 4 μg of the indicated antibody overnight at 4°C, followed by incubation for 4 hr with MagnaChIP Protein Protein A/G magnetic beads (Millipore). Beads were then washed thoroughly with RIPA150, with LiCl – containing buffer and with TE, RNAse-treated for 30 min at 37°, and Proteinase K-treated for at least 2 hr at 56°. De-crosslinking of protein–DNA complexes was performed by an overnight incubation at 65°C. DNA was then subjected to by phenol–chloroform extraction, followed by ethanol precipitation and quantified by real time PCR using specific set of primers for each promoter, listed in Primer table III, previously tested to evaluate their amplification efficiency, and GoTaq qPCR Mater Mix (Promega).

The following antibodies were used for ChIP: anti-H3K4me3 (ab8580, Abcam), anti-acetyl-Histone-H3 (06-599, Millipore), anti Ezh2 (17-662, Millipore), anti-H3K27me3 (ab6002, Abcam). For each antibody, as a negative control, ChIP was also performed with total IgGs.

Immunofluorescence and BrdU detection

Cells were seeded on 24-well primaria plates (~5-10x10^4 cells per well) and, after 3 or 7 days of culture, were fixed with 4% paraformaldehyde (PFA) for 10 min, washed twice in PBS, permeabilized with PBS 1% Triton X-100 three times for 10 min, followed by 1 hr blocking in 2% BSA (Roche) in PBS. Cells were then stained overnight at 4°C with primary antibodies diluted in blocking buffer. Cells were washed with PBS 0.2% Tween 20 and incubated for 1 hr with appropriate secondary

antibodies. Three more washes in PBS 0.2% Tween 20 were performed and then DAPI (Vectashield) or Hoechst 33342 (Life Technologies) were used to stain DNA. In the case of BrdU pulse labeling (10 μM for 20 hours for neonatal, 48 hours for adult cardiomyocytes), following cells permeabilization, DNA denaturation was performed incubating 10 min in 1N HCl on ice and 20 min in 2N HCl at 37°C. To neutralize DNA denaturation, cells were incubated with 0.1 M sodium-borate buffer pH 8.4 12 min at room temperature, then washed three times with PBS 1% Triton X-100. After the neutralization step, the immunofluorescence staining continued as described before with the blocking.

The following were used as primary antibodies: anti-α-sarcomeric actinin (ab9465, 1:250, Abcam), anti-GFP (A6455, 1:200, Invitrogen), anti-BrdU (ab6326, 1:100, Abcam), anti-histone H3 phosphorylated at serine 10 (06-570, 1:100, Millipore), anti-Aurora B kinase (A5102, 1:100, Sigma), anti Ki-67 (PSX1028, 1:100, Monosan).

The following were used as secondary antibodies: goat anti-mouse conjugated to Alexa Fluor 488 (1:500, Molecular Probes), goat anti-rat conjugated to Alexa Fluor 555 (1:500, Molecular Probes), donkey anti-rabbit conjugated to Alexa Fluor 594 (1:500, Molecular Probes).

Image acquisition was performed using an ImageXpress Micro automated high-content screening fluorescence microscope or by manual acquisition of 10 fields/slide, and by counting in a double blinded way, scoring the number of BrdU+, Ki47+, PH3+ or Aurora B+ cardiomyocytes, over the total number of α-actinin+ cells.

Myocardial infarction, echocardiography analysis and AAV in vivo transduction

Myocardial infarction was produced in adult female CD1 mice (8–12 weeks old), by permanent left anterior descending (LAD) coronary artery ligation. Briefly, mice were anesthetized by intraperitoneal injection of ketamine and xylazine, endotracheally

intubated and placed on a rodent ventilator. Body temperature was maintained at 37°C on a heating pad. The beating heart was accessed via a left thoracotomy. After removing the pericardium, a descending branch of the LAD coronary artery was visualized with a stereomicroscope (Leica) and occluded with a nylon suture. Ligation was confirmed by the whitening of a region of the left ventricle, immediately post-ligation. Recombinant AAV vectors, at a dose of 1×10^{11} viral genome particles per animal, were injected immediately after LAD ligation into the myocardium bordering the infarct zone (single injection), using an insulin syringe with incorporated 30-gauge needle. The chest was closed, and the animals moved to a prone position until the occurrence of spontaneous breathing. To evaluate left ventricular function and dimensions, transthoracic two-dimensional echocardiography was performed on mice sedated with 5% isoflurane at 7, 14, 30 and 60 days after myocardial infarction, using a Visual Sonics Vevo 770 Ultrasound (Visual Sonics) equipped with a 30-MHz linear array transducer. M-mode tracings in parasternal short axis view were used to measure left ventricular anterior and posterior wall thickness and left ventricular internal diameter at end-systole and end-diastole, which were used to calculate left ventricular fractional shortening and ejection fraction.

Heart collection and histological analysis

Heart samples were collected at days 7, 14, 30 and 60 days after myocardial infarction. Animals were anaesthetized with 5% isoflurane and then killed by injection of 10% KCl, to stop the heart at diastole. The heart was excised, briefly washed in PBS, fixed in 10% formalin at room temperature, embedded in paraffin and further processed for histology staining, or slowly frozen using isopentane/liquid nitrogen and stored at −80°C until processed for genomic DNA or RNA extraction. Masson's trichrome stainings were performed according to standard procedures, and analyzed for regular morphology and extent of fibrosis. Infarct size was measured as the percentage of the total left ventricular area showing fibrosis.

Production, purification and characterization of rAAV vectors

All the AAV vectors used in this study were generated by the AAV Vector Unit (AVU) at ICGEB Trieste (http://www.icgeb.org/avu-core-facility.html) as described previously [448]. In brief, infectious AAV6 or AAV9 vector particles were generated in HEK293 cells by cotransfecting each vector plasmid together with the packaging plasmid/s expressing AAV and adenovirus helper functions, pDP6 (PlasmidFactory) for AAV6; p5E18 [449] plus helper plasmid (pHELPER; Stratagene) for AAV9. Viral stocks were obtained by $CsCl_2$ gradient centrifugation; rAAV titers, determined by measuring the copy number of viral genomes in pooled, dialyzed gradient fractions, as described previously [450] were in the range of $1x10^{12}$ to $1x10^{13}$ genome copies per milliliter.

In vitro AAV transduction of cardiomyocytes

Cardiomyocytes were transduced with AAV6-EGFP, AAV6-Control, AAV6-sJ1 or AAV6-N1ICD contextually to plating of neonatal cardiomyocytes, at a m.o.i. of $1x10^4$ vg/cell. Adult cardiomyocytes were transduced the day after plating with AAV6-EGFP, AAV6-Control, AAV6-miR-199a, AAV6-miR-590, AAV6-N1ICD, AAV6-sJ1 at a m.o.i of $1x10^4$ vg/cell.

Bisulfite sequencing

Genomic DNA was extracted from day 3, day 7 and adult cardiomyocytes in culture using DNeasy Blood and Tissue Kit (Qiagen). Genomic DNA (1 µg) of each sample was treated for DNA conversion with sodium bisulfite using EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's instruction. Promoter sequences were analyzed with using Methyl Primer Express Software v1.0 (Applied biosystems) to predict CpG islands. Primer sequences were designed using the same software and are listed in Online Table IV. Each primer set was tested to optimize PCR conditions. Amplified fragments were separated on 2% agarose gel, visualized by ethidium

bromide and then purified from the gel using Wizard SV Gel and PCR Clean-up system kit (Promega). The purified DNA was cloned into TOPO T/A cloning kit (Invitrogen). Eight randomly picked clones were sequenced and analyzed using BiQ analyzer software [451].

Statistical Analysis

All data are presented as mean±SEM. Statistical analysis was performed using Prism Software (GraphPad), using 1-way ANOVA followed by Bonferroni post hoc test for the comparison of ≥3 groups.

Table I. Primers used to analyze gene expression levels.

Notch1	Forward	GTGCCTGCCCTTTGAGTCTT
	Reverse	GCGATAGGAGCCAATCTCATTG
Hes1	Forward	GCACCTCCGGAACCTGCAGCG
	Reverse	GCAGCCGAGTGCGCACCTCGGTG
Hey1	Forward	AAAGACGGAGAGGCATCATCG
	Reverse	GCAGTGTGCAGCATTTTCAGG
Hey2	Forward	AGCCCCCATTAACAAGCATTT
	Reverse	TAAGCTAGGGCTCACCAGAGG
Cyclin D1	Forward	AGATTGTGCCATCCATGC
	Reverse	CGGATGATCTGCTTGTTC
Ezh2	Forward	TTGCTAAGAGAGCTATCCAGA
	Reverse	CTGGCTGTATCTGTAATCAAA
sJ1 transgene	Forward	ATTTCTGCTGAAGATATAGCCC
	Reverse	CTCCATTTCATTCAAGTCCTC
N1ICD transgene	Forward	AGCAAGGAAGCTAAGGACC
	Reverse	CTCCATTTCATTCAAGTCCTC
HPRT	Forward	CAGTCAACGGGGGACATAAA
	Reverse	GGGCTGTACTGCTTGACCAA

able II. Primers used for competitive PCR quantification of AAV transduction levels.

Primers for competitor construction		
CMV-β-globin	Forward	CGTCAATGGGTGGAGTATTTGATAACTGCCTTTAACGATG
	Reverse	TGACGTCAATGGGGTGGAGAAGCAGCAATTCTGAGTAGAG
Primers for competitive PCR analysis		
CMV	Forward	CGTCAATGGGTGGAGTATTT
	Reverse	TGACGTCAATGGGGTGGAGA
β-globin	Forward	GATAACTGCCTTTAACGATG
	Reverse	AGCAGCAATTCTGAGTAGAG

Table III. Primers used for chromatin immunoprecipitation.

Notch1	Set 1	Forward	CCCCAGACCTAACTCCAG
		Reverse	ATCAGGTTTTGTGTGTAG
	Set 2	Forward	TGCGCCTGCTACTTTTCGAT
		Reverse	AAATGGTCCCAAGAAGCAACAG
Hes1	Set 1	Forward	ACCAACTCCCTTGTCTCCG
		Reverse	CTCCTCTGCCACTCTCTACCTCTT
	Set 2	Forward	TGTGGGAAAGAAAGTTTGGGAAG
		Reverse	GCTCCAGATCCAGTGTGATCCG
Hey1	Set1	Forward	TCCGCCCTCCCCCTATC

		Reverse	TCACGCTCAGCCTCTGGTTA
Hey2	Set 1	Forward	CGACGTCACACACGCTCACT
		Reverse	GATCTCTCCCGCCAAGTTTCT
	Set 2	Forward	CGACCTAGACGAGACCATCGA
		Reverse	GACACTCTCCCGCTTCTTTGA
Cyclin D1	Set 1	Forward	TTTTCTCTGCCCGGCTTTG
		Reverse	AACTCCCCTGTAGTCCGAGTGA
	Set 2	Forward	GCGAGCCATGCTTAAGACTGA
		Reverse	AATCTCCCTCTGCACGCACTT
GAPDH	Set 1	Forward	TTCCCTGAGTCCTATCCTGG
		Reverse	CTGAGATTGTCCCGCCGAG

Table IV. Primers used for bisulfite sequencing.

Notch1	Set A	Forward	TTAGGGTAGAGTTGGTTTTTGG
		Reverse	ACCCTTACTCCCCTTATAACC
	Set B	Forward	AGGGTGGAATTTTTTTTAAGTG
		Reverse	AAATATACAACCCCATTCACAA
Hes1	Set A	Forward	TTTGATGAAGATGTGGTTAAAG
		Reverse	CAACCTCCCACTAAAATCATAT

	Set B	Forward	AAGTTGGGTAGTTAGGTTGGA
		Reverse	CAATAACCTAAACCCCTCAATT
Hey1	Set A	Forward	GGATAGATTGGGTTTTTTTTAGG
		Reverse	AACAATCCTTCAAAACTTCTCAA
	Set B	Forward	TTTTATTTTTGGGAAGGGG
		Reverse	TCAAAACCTTCCTACACTCAAA
Hey2	Set A	Forward	TATATTTGAGAAGTTGGAGGAATG
		Reverse	ACCAAACCCTCAAAAAATTTTA
Cyclin D1	Set A	Forward	GAGTGGGTTTAGGGTAATTTAGG
		Reverse	ACCAAAATTCCAAAAAAAAAAA
	Set B	Forward	TGGGTAAGTGGTTTTTTGTTTT
		Reverse	CCCCATCTAAAAAACCCTACTT
	Set C	Forward	AGGTTAAGGTTTTTAGGTTTGG
		Reverse	CCAAAATAAATCCCTAAACCTATT
	Set D	Forward	TTTTGAGTTGTTGTTGAGATT
		Reverse	AAAATACAAAAACACCCTATACTTAA
GAPDH	Set A	Forward	TAGTTTTTTGGTTTTTGGGTTT
		Reverse	CTTTTTCACCTAACACTACACAAAA

	Set B	Forward	GGTTGGGGTTTTTTTTTTTAT
		Reverse	CTACCATCCATCACCTAACCTA
GFAP	Set A	Forward	GATGGTTAGGGGTTATGGTTT
		Reverse	CCTTCTCACTTCTACCTCAAAA

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