Regulation of Cardiomyocyte Proliferation by microRNAs and Small Molecules

Thesis

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Regulation of cardiomyocyte proliferation by microRNAs and small molecules

Consuelo Torrini

A Thesis submitted in fulfilment of the requirements of the Open University (UK) for the degree of Doctor of Philosophy

International Centre for Genetic Engineering and Biotechnology
ICGEB, Trieste, Italy

Director of the studies: Prof. Mauro Giacca
External supervisor: Prof Manuel Mayr

Submitted February, 2018
Abstract

Understanding the molecular mechanisms regulating cardiac cell proliferation during the embryonic, fetal and adult life holds a paramount importance in view of developing innovative strategies aimed at inducing myocardial regeneration after cardiac damage. Previous high throughput screening studies in our laboratory identified a series of microRNAs able to trigger cardiomyocyte proliferation and stimulate cardiac regeneration after myocardial infarction.

In the first part of this project, we investigated the mechanism of action of the top ten most effective of these miRNAs, revealing an involvement of the Hippo-YAP pathway in their action. We found that all the investigated miRNAs activated YAP-mediated transcription, nuclear localization of active YAP and increased expression of YAP responsive genes. Of notice, miR-199a-3p, one of the most effective miRNAs exerted its direct effect on two mRNA targets impinging on the Hippo pathway, the inhibitory kinase TAOK1 and the E3 ubiquitin ligase, β-TrCP. Most of the miRNAs inducing proliferation (including miR-199a-3p) also modulated the dynamics of the actin cytoskeleton in the treated cardiomyocytes, which displayed a rounded shape and gross bundles of actin fibers at the cytoplasm periphery. Consistent with these observations, we found that the Cofilin2 mRNA was a direct target of four of the investigated miRNAs and that downregulation of Cofilin2 itself was sufficient to promote cardiomyocyte proliferation, activate nuclear translocation of YAP and stimulate transcription of TEAD-responsive genes.

The second part of the project was aimed at identifying small molecules exerting a mitogenic effect on neonatal cardiomyocytes through an unbiased high-throughput screening (HTS) of a library of 780 FDA-registered drugs. The neuroactive alkaloid harmine was identified as the most powerful molecule at inducing cardiomyocyte proliferation in vitro and heart regeneration after myocardial infarction in vivo. Harmine exerted its activity through the inhibition of the dual specificity phosphorylation-regulated tyrosine kinase, Dyrk1a and, again, the activation of YAP nuclear translocation.

Collectively, these results identify both YAP activation and actin cytoskeleton remodelling as major determinants of cardiomyocyte proliferation and establish the molecular basis for the development of pharmacological therapies to promote heart regeneration through the stimulation of the endogenous capacity of cardiomyocytes to proliferate.
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- the Animal House Facility, and specifically Dr. Serenza Zacchigna and Willy De Mattia;
- Dr Pierluigi Lesizza for performing animal surgery and echocardiography.
**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAV</td>
<td>Adeno-Associated Virus</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>A-V</td>
<td>atrial-ventricular</td>
</tr>
<tr>
<td>BMPs</td>
<td>bone morphogenetic proteins</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>ChIP-Seq</td>
<td>chromatin immunoprecipitation-sequencing</td>
</tr>
<tr>
<td>cKO</td>
<td>conditional knock-out</td>
</tr>
<tr>
<td>CM</td>
<td>cardiomyocyte</td>
</tr>
<tr>
<td>CPCS</td>
<td>cardiac progenitor cells</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>DGC</td>
<td>dystrophin glycoprotein complex</td>
</tr>
<tr>
<td>DPPH</td>
<td>2, 2-Diphenyl-1-picryl-hydrazyl</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene-diamine-tetra acetic acid</td>
</tr>
<tr>
<td>EF</td>
<td>ejection fraction</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ESCs</td>
<td>embryonic stem cells</td>
</tr>
<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G-protein-coupled receptor</td>
</tr>
<tr>
<td>HBV</td>
<td>hepatitis B virus</td>
</tr>
<tr>
<td>HF</td>
<td>heart failure</td>
</tr>
<tr>
<td>HNSCC</td>
<td>head and neck squamous cell carcinoma</td>
</tr>
<tr>
<td>iPSCs</td>
<td>induced pluripotent stem cells</td>
</tr>
<tr>
<td>KO</td>
<td>knock-out</td>
</tr>
<tr>
<td>LV</td>
<td>left ventricle</td>
</tr>
<tr>
<td>LVAW</td>
<td>left ventricular anterior wall</td>
</tr>
<tr>
<td>LVEF</td>
<td>left ventricular ejection fraction</td>
</tr>
<tr>
<td>MAO</td>
<td>monoamine oxidase</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>NDRs</td>
<td>nuclear Dbf2 related kinases</td>
</tr>
<tr>
<td>NMJ</td>
<td>neuromuscular junction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NSCLC</td>
<td>non-small-cell-lung cancer</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PDZ BD</td>
<td>PDZ binding domain</td>
</tr>
<tr>
<td>PE</td>
<td>phenylephrine</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3-kinases</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>rAAV</td>
<td>recombinant adeno associate virus</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA induced silencing complex</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interfering</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
</tr>
<tr>
<td>TAC</td>
<td>Transverse aortic constriction</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate-EDTA</td>
</tr>
<tr>
<td>Tg</td>
<td>transgenic</td>
</tr>
<tr>
<td>TRAP</td>
<td>translating ribosomal affinity purification</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
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1 Introduction
1.1 Introduction to cardiovascular diseases

Therapeutic strategies for cardiovascular diseases have been the challenge of the last century. In fact, cardiovascular diseases are one of the leading causes of death worldwide. In USA only, ischemic cardiomyopathies affects 7.1 millions people and 4.5 millions patients suffer of cardiac insufficiency after myocardial infarction, associated with a mortality rate of more than 50% within the follow-up period of 5 years (Writing Group, Mozaffarian et al. 2016).

Ischemic heart failure, as a consequence of myocardial infarction, occurs when cardiac tissue is deprived of oxygen, due to a coronary arterial obstruction. When the ischemic injury is severe enough to cause loss of critical amounts of cardiomyocytes, a series of sequential pathological events occur, including formation of a non-contractile scar, ventricular wall thinning, overload of blood flow that lead to ventricular remodeling, heart failure and most probably to death (Mahmoud and Porrello 2012).

The therapies currently available for patients with myocardial infarction and heart failure, including treatment with ACE-inhibitors, diuretics and beta-blockers, aim at reducing the impact of fibrotic remodeling, thus improving the morphological and functional impairment of the injured heart. However, none of these therapies are able to solve the real critical problem of an infarcted heart, namely the dramatic depletion of cardiomyocytes causing a severe reduction of cardiac contractility and pump function. Therefore, there is a compelling need to explore alternative approaches and new therapeutic strategies aiming at regenerating the injured heart and increasing its physiological functions.

1.2 Heart regeneration

In the animal kingdom, the regenerative potential is not equally distributed among species. Invertebrates, such as insects, planarians and echinoderms, have a strong regenerative ability. Among vertebrates, Axolotl, zebrafish and newts are great examples of regeneration after damage of many different organs, such as limbs or fins. In mammals, this regenerative potential is very limited and restricted to minor parts of the body. The peculiar ability of vertebrates to regenerate is closely related to their capacity to maintain the developmental transcriptional profile ready-to-use. Understanding the peculiar mechanisms of regeneration remains a great challenge in regenerative medicine, especially to face damages to those tissues with poor proliferative capability, including the heart. In this respects, pioneering studies by Poss and Oberpriller reported examples of complete heart regeneration in salamander and zebrafish after heart resection of the left ventricular apex (Poss, Wilson et al. 2002) (Oberpriller and Oberpriller 1974). Regeneration is due to dedifferentiation of pre-existing cardiomyocytes adjacent to the resected area, a process sustained by sarcomere breakdown and cytoskeletal remodeling. A specific gene expression program drives cardiomyocyte differentiation, driven by GATA4 (Lepilina, Coon et al. 2006) and Hand2 (Schindler, Garske et al. 2014). Of note, the newly formed cardiomyocytes are fully able to functionally integrate into the preexisting myocardium.
Multiple recent evidence has demonstrated that the mammalian heart retains its proliferative potential during embryonic development and early post-natal period, while it rapidly looses it after birth (van Amerongen and Engel 2008). In humans, cardiomyocyte cell cycle withdrawal seems to arise around the first year of life, as concluded from recent findings (Li, Wang et al. 1996, Mollova, Bersell et al. 2013) (Bergmann, Zdunek et al. 2015).

A complete functional rescue of cardiac function after acute myocardial ischemia has been recently reported to occur in a two months-old baby with myocardial infarction (Haubner, Schneider et al. 2016). This evidence further strengthen previously published hypotheses, suggesting the possibility of heart regeneration in children and newborns, as observed in rodents (Macmahon 1937, Saker, Walsh-Sukys et al. 1997). In the adult life, injured myocardial tissue is largely replaced by a fibrotic scar, resulting from the lack of proliferative potential of adult cardiomyocytes. However, a limited capacity of cardiac renewal can still occur in adult animals, as demonstrated by using transgenic mice (Hsieh, Segers et al. 2007), the carbon-dating technique in humans (Bergmann, Bhardwaj et al. 2009) or the thymidine-labelling experiments in mice (Soonpaa and Field 1997). A fundamental question was to identify the cell types involved in this regeneration process. Pre-existing cardiomyocytes were identified as the main source of heart regeneration after injury in a model of apical resection in transgenic cmIc2:EGFP zebrafish (Jopling, Sleep et al. 2010) and in rodents (Hsieh, Segers et al. 2007, Senyo, Steinhauser et al. 2013).

In the rodent studies, cardiac cell turnover was estimated to be of 0.76% per year in young adult mice, which decreases with age. This rate resulted significantly increased (4 folds) in the infarct border zone, after myocardial injury. In humans, the measurement by mass spectrometry of carbon-14 incorporated in the DNA during the Cold War in the atomic weapon-testing period was used as a tool to date human cardiomyocytes. Exploiting this technique, it was estimated a cardiomyocyte renewal of about 1% per year in in young adults, which decreases to 0.45% in aged people (Bergmann, Bhardwaj et al. 2009). Although an innate cardiomyocyte turnover has been detected, this is clearly not sufficient to compensate for the critical cardiac cell loss after myocardial infarction. Therefore, understanding the molecular pathways determining the re-entry of cardiomyocytes into the cell cycle remains a critical goal of current research.

1.2.1 Learning from evolution: filling the gap between amphibians and mammals

1.2.1.1 Evolutionary and developmental adaptations in heart regeneration
Morphological differences in heart among species are results of the adaptation from aquatic to terrestrial life. Since these differences correlate with a decreasing heart regeneration capability, it appears important to understand the morphological and physiological changes occurred during this transitions.
The differences between mammals and amphibians consist not only in cardiac chamber morphology, but also in the presence of a complex coronary vasculature, which is not present in amphibians (Reese, Mikawa et al. 2002, Perez-Pomares, Gonzalez-Rosa et al. 2009). Pressure, temperature, hypoxia, metabolism, immune response and blood clotting all parameters that significantly changed during the transition of life to earth.

1.2.1.2 Pressure
The conquest of terrestrial land forced the evolution to supply animals of a closed circulatory system. In the early stage of mammal life, a significant increase in blood pressure correlated with an increase of mechanical load on ventricular walls (Hillman, Kallapur et al. 2012), resulting in cardiomyocyte adaptive changes, with important consequences at the structural and metabolic levels. Recent evidence sustains the hypothesis that the increased mechanical load in adult human heart is responsible for cardiomyocyte cell cycle arrest, as ventricular unloading stimulates cardiomyocyte proliferation (Canseco, Kimura et al. 2015). According to this notion, mammals might have lost their heart regenerative capability to achieve a more functional pumping heart.

1.2.1.3 Body temperature
Another important factor, correlated to the loss of cardiac regeneration ability in vertebrates, is body temperature. In fact, it is known that neonatal mice, similar to fish and amphibians, do not have a thermoregulatory system. Interestingly, this peculiarity is somehow conserved in human infants, who still are not able to maintain constant the body temperature. Thermoregulation is achieved in adult organisms (Tourneux, Libert et al. 2009). The environmental temperature seems to have an effect in many examples of regeneration. For limb regeneration, newts prefer a warmer environment (Tattersall, Tyson et al. 2012). Also in fish, the time frame necessary for completing fin regeneration seems to be dependent from temperature (Sirbulescu and Zupanc 2010). Finally, an in vivo model of brain injury suggests an enhanced regeneration after cerebral ischemia in hypothermic conditions (Yenari and Han 2013). No specific data are currently available on temperature in cardiac tissue of various organisms, however, it appears reasonable to conclude that the thermoregulatory system, similar to metabolic efficiency, might have resulted as a consequence of an important evolutionary pressure. This might have influenced the loss of regenerative ability.

1.2.1.4 Oxygen concentration
Another key player in tissue regeneration is oxygen tension. Interestingly, the level of cardiomyocyte oxygenation dramatically changes from embryonic to post-natal life and mimics the shift in oxygen concentration happened in evolution when life moved from water to land. Interestingly, species with high regenerative ability live in a hypoxic habitat. Recent studies (Puente, Kimura et al. 2014) (Nakada, Canseco et al. 2017) have raised the possibility that high oxygen tension is involved in the cardiomyocyte cell cycle arrest, due to the induction of the DNA damage response because of reactive oxygen species. Moreover, it is well accepted that the
subpopulation of CMs responsible for cardiac renewal shows low metabolic activity and a gene expression profile typical of the one detected in hypoxic conditions (Kimura, Xiao et al. 2016). Of note, in the mammalian adult heart, the gradual exposure to a systemic hypoxemia reactivates the endogenous regenerative potential, unveiling the relevance of hypoxia in the regenerative process (Nakada, Canseco et al. 2017).

1.2.1.5 Metabolism

Adaptation of cardiac cells to an oxygen-rich environment triggered important metabolic changes. During fetal life, the glycolytic pathway is the main source of ATP. At birth, cardiomyocytes undergo profound metabolic changes resulting in a more efficient strategy to supply energy through the mitochondrial oxidative phosphorylation process (Gong, Song et al. 2015). Despite its higher efficiency, oxidative phosphorylation generates many reactive oxygen species (ROS), which trigger the activation of the DNA damage response and, consequently, the exit of cardiomyocytes from the cell cycle (Puente, Kimura et al. 2014). Gross metabolic changes occur also in case of heart failure. Since the oxygen tension in the case of ischemic heart damage is very similar to neonatal hypoxic conditions, in a mouse model of myocardial infarction a decrease of fatty acid oxidative phosphorylation has been detected parallel to an increase in glycolysis. These changes are theoretically favorable to regeneration, but, despite the decrease in oxidative phosphorylation, no significant changes in the glycolytic pathway are detected, nor a significant regeneration of the injured tissue (Nakada, Canseco et al. 2017). This evidence suggests that, in order to create a favorable environment for cardiomyocyte cell cycle re-entry, the metabolic switch per se is not sufficient, but it requires a complex regulation of different biochemical and biophysical processes, still under intense investigation.

1.2.1.6 Immune system

Another intriguing hypothesis inversely links the tissue regenerative potential along evolution to the development of a mature immune system. In non-mammalian vertebrates a specialized immune system is absent (Buchmann 2014) and, in neonatal mammals, the immune system has very weak pro-inflammatory activity (Sattler and Rosenthal 2016). The absence of the adaptive immune response in amphibians correlates with the great performance in regenerating limbs as if a more permissive immune surveillance would be required to allow regeneration to happen (Epelman, Liu et al. 2015). Of note, key effectors of the regenerative process in zebrafish, amphibians and in neonatal mice are macrophages (Godwin, Pinto et al. 2013) (Lavine, Epelman et al. 2014). Macrophages are massively recruited at the site of heart damage in both neonatal and adults, and are mainly involved in the neoangiogenesis process of the newly-formed tissue, without any direct role in cardiomyocyte proliferation (Lavine, Epelman et al. 2014). Therefore, even if the adaptive immunity is a potent weapon against infectious diseases, it might be detrimental for regeneration, since it is responsible for the localized inflammatory response at the site of injury, which triggers the release of many cytokines, possibly inhibiting cardiac cell proliferation.
1.2.1.7 Blood clotting

The role of blood clotting is a controversial topic in the field of heart regeneration. In some conditions, clotting factors are able to induce regeneration. Some examples are given by mouse liver regeneration and salamander lens: in these cases, platelet activation and other coagulation events are able to start the molecular signaling required for the regenerative response (Lesurtel, Graf et al. 2006). Thrombin is a clotting factor responsible for an opposite effect on lens regeneration in newt and axolotl, although it stimulates newt myocyte proliferation in vitro (Imokawa, Simon et al. 2004). A different molecule (PDGF-BB), secreted by platelets, induces epicardial cells to start neovascularization in a model of heart regeneration in zebrafish (Kim, Wu et al. 2010). However, since blood clotting is not only involved in tissue regeneration but also in repair, the mechanisms involved in these opposite events are still under debate.

1.2.1.8 Structural organization of cardiac myocytes

The contractile protein apparatus of cardiomyocytes greatly differs between mammalian and non-mammalian vertebrates. A recent proteomic study highlights the differences at protein level between adult zebrafish and mouse hearts and their neonatal counterparts (Gomes, Skroblin et al. 2016). The findings confirmed that neonatal hearts share a similar pattern of immature myofilaments, with a limited number of structural proteins, such as Myh6, Myl2 and Myoz2, expressed in mature cardiomyocytes (Gomes, Skroblin et al. 2016). The innate regenerative potential of zebrafish and mouse neonatal hearts is probably associated with an immature sarcomere structure which renders cardiac cells more prone to divide. Actually, it is well assessed that the ability to disassemble sarcomere is required for cardiomyocytes to proliferate (Ahuja, Perriard et al. 2004). Conversely, the structural rigidity of adult mammalian cardiac cells, acquired during adaptation to the increased workload, is a strong physical barrier for cardiomyocyte proliferation and therefore regeneration.

1.2.1.9 Cardiomyocyte nucleation

Another important feature shared by zebrafish, newt and fetal cardiac cells is the mono-nucleation of myocytes (Senyo, Lee et al. 2014). Among mammals, cardiomyocyte binucleation occurs during the first weeks after birth in rodents and at the end of gestation in sheep and humans (Jonker, Zhang et al. 2007). The rate of binucleation in humans is below the average observed in other species and, after birth, cardiac cells get polyploid, a feature of terminally differentiated cardiomyocytes (Mollova, Bersell et al. 2013). The meaning of this process is unclear: it might be due to the rapid growth occurring after birth in mammalian vertebrates, which results in an increased demand of transcriptional activity, connected to higher energetic requirements of the adult life.

The analysis of the regenerative potential through evolution suggests that, in mammals, postnatal normoxic environment triggered a significant increase of ROS production and possibly the activation of the DNA damage response, might be responsible for the cell cycle arrest of adult...
cardiac myocytes. Moreover, structural constrains seem to be greatly involved in the impairment of cardiomyocyte mitosis.

**Figure 1-1 Cardiac regenerative capability in different species**

From zebrafish to humans, heart regenerative potential after injury is significantly diminished. Different pathways lead to recover the damaged tissue in different species. In blue are shown genes promoting heart regeneration, in red those responsible for inhibition. A-V, atrial-ventricular; EMT, epithelial-mesenchymal transition; MI, myocardial infarction. Adapted from (Sahara, Santoro et al. 2015).

1.2.2 Contributors to heart regeneration: all for one and one for all

If the analysis of morphological and physiological changes of the heart along evolution can contribute to find the reason why heart regeneration does not occur in mammals, deep understanding of organogenesis during embryonic life can give important insight as well. The contribution of several cell types in the repair process strictly resembles the series of events occurring during organogenesis. Indeed, regeneration arises from a complex network of signaling networks aimed at organizing cell proliferation, migration, differentiation and functional integration. In the case of myocardial regeneration, four important processes have been reported as key players: angiogenesis, extracellular matrix signaling (ECM), immune response and innervation.

1.2.2.1 Angiogenesis

In the case of organ regeneration, a newly formed network of vessels is required, in order to give nutrients and oxygen supply to the regenerated tissue. This is the case of zebrafish (Lepilina,
Coon et al. 2006) and mouse heart regeneration, following different types of injury (Porrello, Mahmoud et al. 2011): neo-angiogenesis always parallels the tissutal regenerative response. If angiogenesis is impaired, cardiac regeneration fails (Epelman, 2015). Evidence in zebrafish exists showing a crosstalk through the Fibroblast Growth Factor (FGF) between cardiac and endothelial cells of the epicardium (Lepilina, Coon et al. 2006), having the effect of providing an efficient vascular network to the regenerated heart tissue. Several other growth factors are key regulators of neo-angiogenesis in the case of heart regeneration (Zangi, Lui et al. 2013), supporting the important role of the synergistic regulation of different pathways accomplish a complex process such as tissue regeneration.

1.2.2.2 Extracellular matrix (ECM)

Although the involvement of ECM in cardiac regeneration is still poorly understood, it is known that, in the case of myocardial ischemic injury, a massive ECM deposition followed by remodeling is responsible for fibrotic scar formation and, consequently, for impairment of cardiac function. Interestingly, recent evidence suggests a new role for the fibrotic scar, which not necessarily impairs cardiac regeneration. In zebrafish, if scar formation upon injury is impaired by inhibition of TGF-β signaling, heart tissue regeneration is completely blocked (Chablais and Jazwinska 2012). Moreover, since the cellular component of the connective tissue slightly changes during life, it is possible that different cell types make the tissue environment more permissive to the regenerative response (Chablais and Jazwinska 2012). Moreover, the ECM biochemical composition can impact the efficiency of regeneration, acting as a juxtacrine system to induce cardiac cell proliferation (Ieda, Tsuchihashi et al. 2009).

1.2.2.3 Organ innervation

As it happens for the vascular network, a proper innervation of the newly formed tissue is required to an organ in order to successfully accomplish the regenerative process. Recent data have demonstrated that specific pharmacological repression of cholinergic nerve function results in a reduction in cardiomyocyte proliferation in zebrafish and in neonatal murine heart (Mahmoud, O'Meara et al. 2015). Moreover, experimental evidence shows that, in the presence of mechanical denervation of the vagus nerve in different mouse models of myocardial injury, such as apical resection and myocardial infarction, heart fails to regenerate (Mahmoud, O'Meara et al. 2015) (White, Gordon et al. 2015). Interestingly, regeneration can be partially restored by nerve growth factor (NGF) supply, suggesting that NGF is required to sustain heart regeneration.

1.2.2.4 Immune response

In case of myocardial infarction, an acute inflammatory response is activated, causing a series of pathological events resulting in scar remodeling and consequent heart failure. In this context, macrophages are responsible for massive production of pro-inflammatory cytokines, fibroblast activation and ECM remodeling. The immune response is closely linked to regeneration in injured hearts. Recent information from a model of macrophage depletion in neonatal mice has
demonstrated that monocytes and macrophages are required for both regeneration and neo-angiogenesis in the heart (Aurora, Porrello et al. 2014). Of note, these authors found that the absence of macrophages after infarction did not affect cardiac myocyte proliferation directly, but rather the neo-vascularization of the regenerated tissue, because of the lack of pro-angiogenic cytokines.

1.3.1 Cardiomyocyte identity: from transcriptional profile to structural barriers in development and regeneration

A basic requirement for heart regeneration is the reactivation of an embryonic transcriptional program in cardiac cells, to make them dedifferentiate and be permissive to cell cycle re-entry. Importantly, specific gene expression programs characterize each step of cardiac cell differentiation; the transcription factors Nkx2-5 and ISL1, for example, are markers of cardiac progenitors (Ehrman and Yutzey 1999) (Cai, Liang et al. 2003), while Hopx expression characterizes a subpopulation of cardiac committed progenitors, known as cardiomyoblasts (Jain, Li et al. 2015). Indeed, from ChIP-seq studies on the murine embryonic heart at E9.5, emerged that Hopx localized close to genes related to the WNT signaling pathway. Other studies demonstrated that, Hopx promotes SMAD-mediated BMPs signaling and suppresses WNT signaling, enhancing cardiomyogenesis (Jain, Li et al. 2015). During embryonic heart development, GATA4 regulates the final steps of cardiomyocyte differentiation, driving the expression of a-myosin heavy chain and cardiac troponin C (Molkentin, Lin et al. 1997, Gupta, Gemberling et al. 2013). In the case of heart damage, its de novo expression drives de-differentiation of cardiomyocytes, inducing a phenotype permissive for their division, characterized by less tight and de-structured sarcomeres (Jopling, Sleep et al. 2010). This phenotype is the result of deep changes at chromatin level in failing cardiomyocytes: recent evidences have established that dynamic methylation is a key feature during postnatal growth of cardiomyocytes and interestingly the methylation pattern of cardiac cells in case of injury is very similar to neonatal methylation pattern (Gilsbach, Preissl et al. 2014). As it happens for GATA4, the fetal transcription factor Hand2 (the heart and neural crest derivatives-expressed protein 2), is re-expressed during heart regeneration (Schindler, Garske et al. 2014). Conversely, some transcriptional factors are involved in cardiomyocyte cell-cycle exit, as p38 kinase and Meis1. Indeed, Meis1 up-regulation during postnatal life is responsible for the activation of CDK inhibitors (p15, p16 and p21) and cardiomyocyte proliferative arrest (Mahmoud, Kocabas et al. 2013).

It is interesting to note that the peculiar transcriptional profile of cardiomyocytes involved in regeneration leads to a partial cell de-differentiation, mainly affecting the sarcomere structure and the actin cytoskeleton, responsible for the contractility and the rigidity of the mature cardiac cells (O’Meara, Wamstad et al. 2015).
1.3.2 Regulation of cardiomyocyte proliferation

Several cell-cycle regulators are expressed during heart development and downregulated in the adulthood (reviewed in (Xin, Olson et al. 2013)). Genetic manipulation of these regulators promotes, to different extents, de-differentiation and proliferation of cardiac cells.

1.3.2.1 FGF1 and inhibition of p38 pathway

The p38 kinase belongs to the MAP kinase family and is a negative regulator of the cell cycle. The inhibition of p38 results in neonatal cardiomyocyte proliferation, while its overexpression has an opposite effect, blocking cardiac cells proliferative ability (Engel, Schebesta et al. 2005). In addition, it was observed that a combination of p38 inhibitors and FGF1, fibroblast growth factor 1, increases mitotic events with an improvement of heart function in a mouse model of myocardial ischemia, due to the impairment of apoptosis at the site of injury (Engel, Hsieh et al. 2006). FGF1 also promotes new vessel formation, while p38 inhibition is associated to the expression of genes involved in cell cycle regulation. However, despite these evidences, the increase in cardiac cell proliferation remains modest.

1.3.2.2 Neuregulin-1 signaling

Neuregulin1 (NRG1) is a growth factor involved in cardiac cell proliferation during embryonic life. NRG1 shows an agonist effect on the ErbB receptor family. It exerts its function binding ErbB4, inducing either ErbB2-ErbB4 or ErbB4-ErbB4 dimerization. Of note, NRG1 is able to induce proliferation only in mononucleated cardiac myocytes (Bersell, Arab et al. 2009). In adult cardiac myocytes, NGR1 treatment induces sarcomere breakdown and an immature sarcomere structure similar to the fetal one. Moreover, NGF1 not only promotes cardiomyocyte proliferation in adult hearts through PI3K activation, but its activation is also required during development for the complete maturation and final differentiation of CMs, under the control of BMP10 (Grego-Bessa, Luna-Zurita et al. 2007).

1.3.2.3 Notch signaling

In Vertebrates, Notch signaling controls mesodermal commitment during development, playing a critical role in mammalian embryonic cardiogenesis; mice lacking the genes encoding either Notch receptors or their ligands show early embryonic lethality, mainly because of cardiovascular abnormalities (Penton, Leonard et al. 2012). In addition to its essential role in heart development and cardiac specification during embryogenesis, Notch regulates proliferation of immature cardiomyocytes during fetal and post-natal life (Collesi, Zentilin et al. 2008) (Campa, Gutierrez-Lanza et al. 2008) (Croquelois, Domenighetti et al. 2008) and is essential for the maintenance of the heart structural and functional integrity after myocardial infarction (Kratsios, Catela et al. 2010) during the response to increased workload or during heart failure (Croquelois, Domenighetti et al. 2008) (Oie, Sandberg et al. 2010). In the species that efficiently regenerate the heart after damage, Notch is essential for postnatal cardiomyocyte proliferation (Raya, Koth et al. 2003) (Zhang, Han et al. 2013), while recent evidence indicates that it is largely ineffective in
driving cardiac regeneration in adults, because of permanent epigenetic modification at main Notch-responsive promoters (Collesi, Zentilin et al. 2008) (Felician, Collesi et al. 2014).

Among the pathways described as capable to stimulate cardiomyocytes proliferation, the YAP-Hippo signaling pathway is considered the most potent driver in the regulation of cardiomyocyte proliferation.

1.4. A path for regeneration: Hippo Signaling Pathway

The Hippo Pathway was discovered through a genetic screening in Drosophila aimed at identifying genes involved in the control of organ size (Xu, Wang et al. 1995) (Justice, Zilian et al. 1995). It is a highly conserved pathway, involved in the maintenance of tissue integrity through a intracellular signaling cascade whose inactivation, through genetic deletion of proteins such as Warts (Wts), Hippo (Hpo), Salvador (Sav) and Mats (Mts), results in a common phenotype of massive proliferation and an overgrowth of the interested tissue (Halder and Johnson 2011).

In the past decade, the Hippo signaling pathway has been described to be involved not only in tissue growth, but also in stem cell pluripotency and cell fate determination (Lian, Kim et al. 2010, Qin, Hejna et al. 2016, Britschgi, Duss et al. 2017). Moreover, it is involved in the control of the balance between proliferation and apoptosis, often associated to the development of solid cancer (Low, Pan et al. 2014).

Actually, solid cancer cells often show a hyperactivation of the Yes-associated protein (YAP), which, together with TAZ, is the main regulator of the Hippo signaling cascade (Zhao, Li et al. 2010). Since proliferation and apoptosis are key regulatory processes in both regenerative medicine and oncology, targeting this pathway has become an attractive clinical strategy for the development of new therapeutics.

The Hippo transduction cascade has a high level of molecular complexity and redundancy, which reflects the importance of this signaling pathway in the regulations of critical cellular processes. Briefly, in humans, the STE20-like protein kinase 1/2 (MST1/2) (Hippo in Drosophila) was described as an upstream kinase, which phosphorylates large tumor suppressor homolog 1/2 (Lats1/2) (Warts in Drosophila); these two kinases are able to directly interact through to the adaptor protein Sav1 (Salvador). MOB1A and MOB1B (Mats in Drosophila) enhance the kinase activity of Lats1/2. Lats1/2 are kinases able to directly regulate YAP/TAZ, the final effectors of the Hippo transduction signaling; upon activation, Lats1/2 phosphorylates the transcriptional co-activator, Yes-associated protein YAP, and its paralogues PDZ-binding domain, TAZ (Yorkie in Drosophila). YAP phosphorylation on Ser127 has two major consequences: it stimulates its binding to the 14-3-3 protein family, preventing YAP translocation to the nucleus and it triggers phosphorylation of CK1d and the recruitment of beta-TRCP, resulting in YAP/TAZ proteasomal degradation (Hariharan 2006, Zhao, Li et al. 2010).
When the MST1/2 kinase cascade gets inactivated, YAP is no longer phosphorylated and translocates into the nucleus to activate the transcription of target genes, including CTGF, CyR61 and Birc5 (Zhao, Ye et al. 2008) (Shimomura, Miyamura et al. 2014).

Since YAP/TAZ are not able to directly bind DNA, their function is mediated by several transcription factors; among these are TEAD1-4 (TEA domain family member- Scalloped in Drosophila) (Zhao, Ye et al. 2008), Runx (Zaidi, Sullivan et al. 2004), FoxO1 (Shao, Zhai et al. 2014), Tbx5 (Murakami, Nakagawa et al. 2005) and SMADs (Ferrigno, Lallemand et al. 2002) but their role in Hippo signaling activation is still not completely understood. The canonical inhibition of the Hippo cascade leads to TEAD1-4-mediated gene expression, preferentially repressed by VGLL4 repressor binding, which is lost in the presence of the YAP/TAZ co-activators (Koontz, Liu-Chittenden et al. 2013).

Consistently, mouse models of knockdown of genes representing the active core of the Hippo pathway, such as MST1/2, SAV1, MOB1A/B or LATS1/2, showed a significant increase of TEAD target gene expression, similar to YAP overexpression models (Dong, Feldmann et al. 2007) (Camargo, Gokhale et al. 2007, Lee, Lee et al. 2010) (Nishio, Hamada et al. 2012).

Downstream target genes of YAP/TAZ signaling were first identified in Drosophila: among them, cyclinE, diap1 and the microRNA bantam are all mediators of cell proliferation and survival (Wu, Liu et al. 2008). Other targets are p73 and ErbB-4; the former is a transcription factor involved in the stimulation of the apoptotic response and in DNA-damage signaling in association with YAP (Downward and Basu 2008); ErbB-4 is a tyrosine kinase receptor, which is able to induce a mitogenic signalling after YAP-mediated transactivation (Komuro, Nagai et al. 2003, Schuchardt, Bhat et al. 2014).
phosphorylation of the final effector of the signaling pathway, YAP, to its consequent cytoplasmic retention or proteasome-dependent degradation. Conversely, when the pathway is turned off, YAP is no longer inhibited, it translocates into the nucleus and, in association with the TEAD transcription factors, induce cell cycle gene expression.

**1.4.1 YAP and TAZ protein structure**

Both YAP and TAZ show a very conserved protein structure. Both of them show a coiled-coiled and PDZ-binding domain, essential for nuclear translocation (Shimomura, Miyamura et al. 2014). Actually, it was found that the protein zona occludents 2 (ZO2) can bind this region and regulate YAP nuclear translocation via recruitment of some other regulators, or inducing conformational changes, critical for YAP activation (Oka, Schmitt et al. 2012).

![Figure 1-3 Schematic representation of YAP and TAZ structure](image)

The functional domains of YAP/TAZ are represented, together with interacting proteins. The PDZ binding domain (PDZ BD) is located at the C-terminus, while the WW domains are in the central region. The TEAD binding domain allows YAP and TAZ to exert their function as transcription factors. The serine residue phosphorylated by upstream protein kinases are indicated in dark yellow, while those target of CK1 phosphorylation are in a lighter yellow; the c-Abl binding site is in light blue. The lysine residue target of Set7 methylation is in pink. Adapted from (Piccolo, Dupont et al. 2014).

Moreover, both YAP and TAZ share other two important motifs: a WW domain in the central region of the protein and a transactivation domain in the C-terminal region (Fig.1-3). YAP only shows two interesting domains: the proline rich-domain at N-terminus mediates its interaction with the heterogeneous nuclear ribonucleoprotein U (hnRNP U), while the SH3-binding motif, close to the second WW region, mediates its binding with the Yes tyrosine kinase (Sudol 1994,
The phosphorylation of YAP by LATS on the five serine residues is one of the best-described modifications to regulate YAP activity. The S127A mutation was observed to increase YAP localization inside the nucleus (Basu, Totty et al. 2003). Phosphorylations on Ser127 of YAP and on Ser89 of TAZ create docking sites for 14-3-3 proteins, which promote their cytoplasmic retention (Yu, Zhao et al. 2012) (Basu, Totty et al. 2003). Conversely, phosphorylation on Ser381 has a different functional role, leading to YAP proteasomal degradation (Kim, Kim et al. 2013). Several additional post-translational modifications can occur on YAP/TAZ, such as acetylation, methylation and sumoylation; however, the relevance of most of these modifications is still not completely understood.

1.4.2 YAP signaling pathway: upstream activating stimuli

Among the recently identified proteins involved in the direct regulation of YAP1, the NDR (nuclear Dbf2 related kinase) family members are able to inhibit YAP1/TAZ activation promoting their cytoplasmic retention (Zhang, Tang et al. 2015). NRD1/2 kinases, including LATS1/2, are AGC serine/threonine kinases (STK38/STK38L), which are characterized by a highly conserved NTR domain, the N-terminal regulatory portion, and the functional kinase segment, located between the subdomain VII and VIII (Hergovich, Stegert et al. 2006). NDR1/2 are able to directly interact with MOB through their NTR domain, however different MOB isoforms preferentially interact either with NDR1/2 or with LATS1/2. Actually, MOB2 preferentially binds NDR2, whereas MOB1A/B interact with both NDR1/2 and LATS1/2. Interestingly, their upstream activation can be mediated by MAPK4 and MST kinases via phosphorylation on Thr444/Thr442 (Stegert, Hergovich et al. 2005) (Hergovich, Kohler et al. 2009). NDR proteins can be functionally regulated by several mechanisms, including phosphorylation or changes in subcellular distribution. Even though the absence of NDR1/2 is associated with a strong increase of YAP activity, their role in the physiological cellular context is poorly understood.

Other kinases have been described to modulate the Hippo pathway, such as the MAP4K family and TAOK1/3, which mediate LATS1/2 activation through phosphorylation (Meng, Moroishi et al. 2015) (Boggiano, Vanderzalm et al. 2011). Of note, TAOK activation can affect the Hippo transduction cascade at two different levels: upstream of MST1/2, mediating their phosphorylation and in parallel to MAP4 kinases and MST1/2, acting directly on LATS1/2 activation (Plouffe, Meng et al. 2016). Moreover NF2, neurofibromatosis type 2, is involved in the activation of LATS1/2, carried out by MAP4Ks and TAOKs. Indeed, NF2 ablation triggers a more relevant decrease of YAP phosphorylation than the absence of MST1/2 (Plouffe, Meng et al. 2016). NF2, is a protein belonging to the FERM domain protein superfamily, whose expression is very abundant in the “zonula adherens”, responsible, together with tight junctions, for tissue architecture and integrity (Lallemand, Curto et al. 2003). In this peculiar cellular microenvironment, NF2 and other proteins such as AMOT, Expanded and α-Catenin, are also
involved in the regulation of the Hippo signaling cascade, recruiting LATS at the junctions and consequently inhibiting YAP.

Several different stimuli can modulate the Hippo pathway through G-protein-coupled receptor activation (GPCRs), a class of cell surface receptors able to influence a plethora of biological functions. Due to the different G-proteins, which can be recruited by these receptors, GPCRs are able either to promote or block the Hippo-signaling pathway. In fact, G12/G13, Gq/11 and Gi-coupled receptors inhibit LATS1/2 function, resulting in a positive effect on cell proliferation coherent with YAP activation, while Gs-coupled receptors activate LATS1/2 kinases. Furthermore, Ga proteins exert their effect on Rho GTPases and cytoskeleton remodeling, but how this is linked to LATS activity remains quite obscure (Yu, Zhao et al. 2012).

**1.4.3 YAP/ TAZ as mechanosensors**

As previously discussed, ECM properties and mechanical cues assume a fundamental role in organ structure development, by influencing cellular transcriptional profiles in adult tissues. At the cellular level, this implies a tight regulation of intra- and extracellular signals, resulting in a dynamic remodeling of actin cytoskeleton. Any alteration of the mechanical forces on cells can actively contribute to the development of pathological conditions such as cancer transformation or fibrosis.

Despite the relevance of cellular mechanoresponse is widely recognized, little information is available on the mechanism of intracellular transduction of these stimuli. In this context, our current knowledge on YAP/TAZ function upon mechanical inputs is also shedding lights on cell mechanobiology.

Actually, YAP and TAZ are mainly regulated by their localization; stiffness of the extracellular matrix or cytoskeletal tension are responsible for their nuclear distribution, which represents their functional activation, while soft substrates induce YAP and TAZ retention inside the cytosol (Aragona, Panciera et al. 2013). Actin cytoskeleton is essential for YAP/TAZ mechanotransduction: indeed, the inhibition of actin polymerization results in YAP/TAZ nuclear exclusion (Dupont, Morsut et al. 2011). These findings have been further supported by evidences obtained using F-actin inhibitory proteins, as CAPZ, ADF/Cofilin, demonstrating YAP activation upon a specific disposition of polymerized actin (Aragona, Panciera et al. 2013). Interestingly, the link between YAP/TAZ activation and changes in cellular geometry seems to be Hippo-independent, implying alternative regulations of YAP/TAZ signaling (Wada, Itoga et al. 2011).

FAK, SRC and RHO proteins have been described as molecular links between the extracellular signals (either from other cells or from the ECM) and the intracellular environment, possibly leading to YAP/TAZ activation. The YAP response to mechanical cues is to generate intracellular forces; this implies the formation of contractile actomyosin structures leading to cytoskeleton reorganization. Since in the heart several mechanical cues together with important cell-contact interactions are responsible for tissue integrity and myocardial functionality, the Hippo pathway holds a fundamental role in cardiac development as well as in heart homeostasis.
1.4.4 YAP signaling: between cancer and regeneration

YAP/TAZ involvement in regulation of cell proliferation makes them interesting targets for therapeutic strategies in oncology and regenerative medicine. In non-small-cell-lung-cancer (NSCLC), patients showing high expression of YAP/TAZ have a poor prognosis; however, even though YAP/TAZ presence is essential for malignant progression, it is not sufficient to promote the development of NSCL cancer, as well as glioblastoma (Bhat, Salazar et al. 2011). In other types of malignancies, as in colorectal and liver tumors, YAP is required for malignant transformation; in colon and lung cancers arising from K-RAS mutation, both YAP and K-RAS regulate the EMT-related genetic program (Shao, Xue et al. 2014). Moreover, K-RAS is able to promote YAP transcriptional activation, therefore supporting the concept of a Hippo-independent mechanism.

Intriguingly, elevated YAP levels provide several advantages to cancer cells, including enhanced resistance to chemotherapeutic treatments, as observed in breast cancer (reviewed in: Harvey, Zhang et al. 2013). Moreover, recent findings demonstrate that YAP/TAZ activation, in certain cases, sustains the resistance to oncogene-targeted therapy; elevated levels of YAP correlate with a poor response to RAF and MEK inhibitors in treatment cancer histotypes bearing a BRAF-mutation, acting as a survival stimulus for tumor cells (Lin, Sabnis et al. 2015). Since cell cycle regulation is a crucial process not only in tumor development, but also for organ regeneration, the role of YAP was explored in this context as well. Indeed, the regenerative process occurring in mammalian liver upon different kinds of injury is mediated by Hippo pathway inactivation, specifically by inhibition of Mst1/2 and Lats1/2, associated with a GA-binding protein-dependent YAP overexpression (Wu, Xiao et al. 2013). Neuromuscular junction (NMJ) regeneration is also mediated by YAP activity. Defective NMJ formation was observed in YAP KO mice, with deficit in pre- and postsynaptic distribution and size (Zhao, Shen et al. 2017). Furthermore, YAP absence inhibits muscle re-innervation, again highlighting the fundamental role of YAP in NMJ regeneration. Interestingly, YAP-/− mice showed a decrease in β-catenin levels, suggesting a role of WNT signaling in NMJ regeneration. However, induction of WNT signaling in YAP-/− mice ameliorated, but not completely rescued, the de novo formation of NMJ, demonstrating that β-catenin is not the only player downstream of YAP-mediated NMJ regeneration (Zhao, Shen et al. 2017).

Intestine regeneration requires YAP functional activity too. In fact, a decreased level of cell proliferation was observed in the crypts of YAP KO mice, demonstrating that YAP regulation of intestinal stem cell cycle affects intestinal regeneration. Besides the role of YAP in the regenerative gut response after damage (Gregorieff, Liu et al. 2015) (Barry, Morikawa et al. 2013), the involvement of WNT signaling in intestinal regeneration is commonly accepted.

Another example of the pivotal role of YAP in the regeneration process is bone regenerative response. A dichotomous behavior of YAP in chondrocyte differentiation was described (Deng, Wu et al. 2016). Indeed, YAP is both responsible for early chondrocyte proliferation, through TEAD mediated Sox6 expression, and block of chondrocyte maturation, due to Col10a1
expression inhibition, mediated by Runx2. In addition, YAP drives the early phases of fracture repair, through the suppression of the development of cartilaginous callus tissue. Finally, YAP was identified as a key player in cardiac regeneration, as discussed below in details.

1.4.5 Hippo pathway: the heart issue

Heart development is a complex process requiring the integration of spatial-temporal signaling pathways and the interaction of different cell types (Brade, Pane et al. 2013). A network of interconnected signaling pathways flow into the nucleus, triggering the activation of a specific set of transcription factors, among which is Tbx5, a major regulator of the gene expression program leading to heart development. The finding that Tbx5 is activated by YAP raised the intriguing possibility that the Hippo pathway might be involved in embryonic cardiac development (Murakami, Nakagawa et al. 2005). Several studies have been performed to better understand the role of the Hippo kinase cascade in the heart. Data suggest that a deficiency of the Hippo pathway during heart development causes cardiac overgrowth; in fact, selective cardiac inactivation of Mst1/2, Sav1, and Lats2, which negatively affect YAP activation, led to marked myocardium enlargement due to an increase in cardiomyocyte number and trabecular expansion (Heallen, Zhang et al. 2011).

The role of YAP in cardiac growth has been investigated using either gain or loss-of-function models (Xin, Kim et al. 2013) (von Gise, Lin et al. 2012). YAP1 activation is fundamental and sufficient to induce proliferation of embryonic cardiomyocytes and is also responsible for the physiological enlargement of the heart during development, via hyperplastic more than hypertrophic growth. These findings suggest that YAP is sufficient to promote cardiomyocyte cell cycle activity. On the contrary, YAP cardiac-specific inactivation, in early stages of the heart development (at E10.5), is lethal, because of a marked reduction of cardiomyocyte number and dramatic hypoplasia (Xin, Kim et al. 2011). Of note, in the heart, YAP signaling is transduced by canonical transcription factors, TEAD1-4, which are fundamental for YAP mitogenic activity in cardiomyocytes (Wu, Liu et al. 2008) (Zhao, Ye et al. 2008). Indeed, lack of expression of TEAD1 results in E11-12 embryonic death, a critical time frame for heart development. Conversely, TEAD1 overexpression in the heart during postnatal period promotes cardiac pathological remodeling, responsible for heart failure (Chen, Friedrich et al. 1994).

Mice overexpressing TEAD1 show age-dependent cardiac dysfunction, associated with an increase in GSK3β activation associated with decrease levels of both nuclear β-catenin and NFATc3/c4 (Tsika, Ma et al. 2010). These studies show that increased TEAD1 can promote the development of heart defects, typically present in cardiac remodeling following heart failure.

Since, in mammals, both YAP and its paralogue are present, TAZ cardiac-specific KO mice were generated. Surprisingly, postnatal cardiac TAZ deletion did not show any specific phenotype and did not alter mouse lifespan, demonstrating that the role of TAZ in heart development and function is dispensable, compared to YAP (Xin, Kim et al. 2013).

Cardiac-specific Mst1 overexpression sustains a massive cardiomyocyte apoptosis, responsible
for animal death due to dilated cardiomyopathy. Indeed, a dominant-negative MST1 protein exerts a protective function against remodeling in a model of MI (Yamamoto, Yang et al. 2003). Besides its critical role during heart development, Yap exerts a critical function in heart regeneration upon injury. In a mouse model of myocardial infarction, increased levels of YAP not only prevent cardiac cell loss but also fibrotic scar formation. Consistently, constitutive activation of YAP1 is able to extend the regeneration window, triggering an improvement of cardiac function (Xin, Kim et al. 2013). In a heart-inducible YAP transgenic mouse model, in case of myocardial infarction, YAP activation significantly reduced scar size, with a net improvement of myocardium functionality. These effects were due to adult cardiomyocyte cell cycle re-entry, driven by YAP activation. Of note, sustained expression of YAP triggered significant downregulation of specific genes associated with terminal differentiation of cardiac cells (such as muscle- and oxidative metabolism-related genes), while cell cycle gene expression increased, suggesting that YAP induces a genetic program leading to a less differentiated phenotype, in order to make proliferation possible (Xin, Kim et al. 2013). Hippo signaling deficiency leads to increased cardiomyocyte proliferation and myocardial protection after MI. In fact, Sav1 KO mice show improved cardiac function and smaller scar size after MI (Heallen, Morikawa et al. 2013). These findings suggest that YAP is fundamental for adult heart homeostasis and has a strong cardioprotective function, probably due to its ability to promote both cardiac cell survival and proliferation in response to injury. Recent literature shows a strong interconnection between Yap signaling and other intracellular signal transduction pathways, acting synergistically in the regeneration process (Shao, Zhai et al. 2014) (Lin, Zhou et al. 2015).

Several published studies have analyzed the complex role of YAP in cardiac regeneration and have thus expanded our knowledge on the interaction of YAP-Hippo signaling with other intracellular signal transduction pathways and with the different cellular processes occurring after injury.

An interesting study was performed in conditional cardiac specific Salvador KO mice bearing a dystrophin loss-of-function (Mdx background), in order to explore the link between the Hippo pathway and the dystrophin glycoprotein complex (DGC) (Morikawa, Heallen et al. 2017). Both Salv KO, and Salv;Mdx DKO mice were able to regenerate the apex after heart apical resection. Interestingly, DKO mice were able to repair the resected heart showing an impressive cardiac overgrowth, often resulting in the formation of a second apex. These findings suggest that the Hippo pathway and DGC proteins are both responsible to restrict cell growth after damage, suggesting a key role of the DGC in arraying proliferating cardiomyocytes in a 3D space. When the same mice were analyzed in a TAC model of hypertrophy, the Salv; Mdx DKO myocardium showed milder cardiac fibrotic features than the mdx control mice, associated with a contained dilatation and physiological parameters similar to the Salv KO controls. Moreover, both Salv KO and DKO shared an increased level of nuclear YAP, demonstrating that the cardiomyopathy of mdx mice was abolished by Salvador depletion, resulting in YAP activation, with a consequent boost of cardiomyocyte proliferation and protection from apoptosis (Morikawa, Heallen et al.
The use of innovative tools, as translating ribosomal affinity purification (TRAP), has allowed the recovery and analysis of cardiomyocyte transcripts in several mouse models. Cardiomyocytes purified from Salv-depleted infarcted hearts express pro-proliferative genes, as well as genes related to vasculogenesis (FGFs and EGFs) and, more importantly, stress-related genes. Among these genes there is Parkin2 (Park2), involved in the quality control of mitochondria, which was also described to be important in cardiac repair, suggesting the need of mitochondrial quality control in the cardiac regenerative process. Park2 is involved in mitochondrial maturation and plays a key role in the cardiomyocyte metabolic transition from the embryonic to the adult age (Dorn 2016). Its absence in Salv KO mice negatively affected heart function, suggesting that Park2 is required not only for myocardial function but also for the resolution of the fibrotic scar (Leach, Heallen et al. 2017).

Recent studies have identified multiple molecular mechanisms exploited by YAP to control heart growth and cardiomyocyte proliferation. In Sav1 KO mice, Sav inactivation triggers β-catenin translocation into the nucleus leading to up-regulation of WNT pathway target genes (Heallen, Zhang et al. 2011). Consistent with these findings, YAP overexpression in fetal hearts caused an increase of WNT signaling (Xin, Kim et al. 2013).

Insulin-like growth factor (IGF) signaling is involved in Yap-dependent cardiomyocyte proliferation as well. Indeed, in a model of YAP constitutive activation, increased levels of IGF1 receptor were detected, with consequent GSK3β functional inhibition. Inactivation of GSK3β stabilizes β-catenin, a final effector of the WNT pathway, resulting in the activation of pro-proliferative genes (Xin, Kim et al. 2011). Moreover, YAP activation in neonatal cardiomyocytes resulted in upregulation of cell cycle regulators such as cyclin D, demonstrating a direct link between cell cycle genes and YAP itself (von Gise, Lin et al. 2012).

Recently, the PI3K-AKT signaling pathway was described to cooperate with YAP-signaling in the control of cardiac cell growth and survival. Through a genome-wide screening approach aimed at identifying genes responsible for YAP-induced pro-proliferative effects, it was found that YAP induces the expression of Pik3cb, the phosphatidilinositol-3-phosphate kinase catalytic subunit which is highly expressed during embryonic development and drastically decreases in adulthood. Interestingly, the pattern of expression of Pik3cb superimposes to the one of YAP itself. Strong in vivo evidence supports the requirement of YAP to promote PI3K-AKT mediated cardiomyocyte proliferation. AAV-mediated Pik3cb overexpression in YAP cKO mice after MI triggered a net increase in cardiac function, inducing a net cardiomyocyte proliferative response, paralleled by a reduction of apoptosis (Lin, Zhou et al. 2015).

Collectively, multiple evidence unequivocally demonstrates the pivotal role of YAP as a master gene in cardiac biology. Its effect lasts from embryonic to adult life, since it regulates fetal heart development, induces proliferation in neonatal and adult cardiomyocytes and strengthens the regenerative potential in adult hearts after ischemic injury. Given its central role in heart
regenerative biology, the Hippo-YAP signaling pathway is an attractive target for heart regenerative therapies.

1.4.6 microRNAs targeting YAP1

Cellular processes need a multilayered regulation in order to be straightly modulated. Among the adopted strategies for post-transcriptional regulation of gene expression, microRNAs exploit a pivotal role, given the pleiotropic effect of their action. Several microRNAs have recently been involved in the regulation of the Hippo pathway cascade, mainly as key regulators of tumorigenic transformation. An example is miR-135b in lung cancer, responsible of the metastatic phenotype, given its property to target LATS2, MOB1B and NDR2. Thus, the use of an antagonir, which suppresses miR-135b expression, reduces metastasis and tumor growth (Lin, Chang et al. 2013). Similarly, overexpression of miR-31, a commonly upregulated miRNA in several tumors (Liu, Sempere et al. 2010), is also able to target LATS2, directly binding its 3’UTR as observed in endometrial cancer cell lines (Mitamura, Watari et al. 2014).

Other important miRNAs targeting Hippo pathway members belong to the miR-130 family, whose overexpression has been observed in different cancers, such as bladder, gastric cancer and glioblastoma (Zhu, Wang et al. 2015, Egawa, Jingushi et al. 2016). Of note, YAP exerts a positive feedback on miR-130 expression, which in turn represses VGLL4, an inhibitor of YAP-TEAD complex in hepatocellular carcinoma (Shen, Guo et al. 2015). Interestingly, miR-130a displays a similar function of the fruitfly miRNA bantam, which inhibits the homolog of VGLL4 SdBP/Tgi, further unveiling the evolutionary conservation of YAP molecular signaling. Moreover, upregulation of mir-130b preserves cancer stem cells in glioblastoma, targeting MST1 and SAV1 (Zhu, Wang et al. 2015). On the contrary, both miR-195-5p and miR-186 seem to target YAP with beneficial effects in tumor suppression, specifically on colorectal and hepatic cancer, respectively (Ruan, He et al. 2016, Sun, Song et al. 2017).

Other important insights into the regulation of YAP signaling by miRNAs derived from findings in hepatocellular carcinoma (Liu, Zhang et al. 2015). PreS, a HBV transactivator protein, is able to induce TAZ overexpression by suppressing miR-338-3p. Thus, downregulation of this miRNA, in the presence of preS, causes a reduction in TAZ suppression, resulting in TAZ-mediated promotion of cancer growth (Liu, Zhang et al. 2015). Recently, miR-125a was described to target TAZ in glioblastoma and miR-141 in gastric cancer (Yuan, Xiao et al. 2015, Zuo, Zhang et al. 2015).

A different example is the miR-29 family, upregulated upon YAP overexpression. In this case the pro-proliferative effect is achieved by targeting the 3’UTR of the PTEN transcript. Since PTEN is responsible for inhibition of mTOR, downregulation of PTEN results in mTOR activation and induction of cell proliferation (Tumaneng, Schlegelmilch et al. 2012).
1.5. Gene therapy to promote heart regeneration

Several strategies have been proposed to promote cardiac regeneration. Great excitement was raised in the first decade of this century by the possibility that cells from various derivation might perform as stem cells for cardiac regeneration. This was the case of c-kit-positive cells from the bone marrow (Orlic, Kajstura et al. 2001) and the heart itself (Beltrami, Barlucchi et al. 2003), mesenchymal stromal cells from the bone marrow (Hatzistergos, Quevedo et al. 2010) and heart-derived cardiosphere-forming cells (Smith, Barile et al. 2007). However, when all these cell types have been used for clinical experimentation, the results achieved turned out to be highly disappointing. To date, there is no proof that any cell type with putative stem cell capacity, once injected into the heart, induces real cardiac regeneration (commented in: (Eschenhagen, Bolli et al. 2017). In most instances, implantation of exogenous cells into the heart provides a modest and transitory beneficial effect through the paracrine secretion of factors improving cardiac function, preventing cardiomyocyte apoptosis or inducing neoangiogenesis.

Generation of new myocardial tissue can instead be achieved by the implantation of cardiomyocytes obtained in vitro from ES or iPS cells, as originally shown by the Murry laboratory (Kadota, Pabon et al. 2017). This approach, however, requires the generation of at least one billion cardiomyocytes in culture and their bulk implantation in vivo, with consequent problems related to the electrical and mechanical integration of these cells with the surviving myocardial tissue. An alternative approach would consist in the stimulation of the endogenous capacity of myocardial myocytes to undergo division, therefore achieving endogenous cardiac regeneration. This goal might be achieved by gene therapy.

Gene therapy exploits the idea of modifying cell behavior by delivering nucleic acids. Early attempts of cardiac gene transfer started in ‘90s, with the delivery of angiogenic genes through adenoviral vectors, able to transduce cardiomyocytes at high efficiency and to express high levels of the encoded transgenes. Despite preliminary positive results, most of these studies were prematurely terminated due to the intense inflammatory response and immune rejection elicited by adenoviral vectors (Hedman, Hartikainen et al. 2011). Subsequent attention of the gene therapy community thus turned to the Adeno-Associated Virus (AAV) as a powerful tool for cardiac gene transfer, due to the peculiar tropism of this virus for post-mitotic cells, including cardiomyocytes and the long term episomal persistence of its genome in the infected cells (reviewed in (Zacchigna, Zentilin et al. 2014)). Over 100 clinical trials with AAV vectors have been performed to date (http://www.abedia.com/wiley/), of which one for a cardiac application (AAV1-Serca2a for heart failure (Hulot, Salem et al. 2017).

While the use of viral vectors is essential to transfer large genes into the heart, shorter nucleic acids could be delivered through simple chemical methods. This is the case of the delivery of short interfering RNAs (siRNAs), miRNA mimics and inhibitors, which are currently largely used in RNAi-based therapeutics (Zhou, Zhang et al. 2014) (Fig.1-4). Indeed, microRNAs might also play a role in cardiac regeneration (Eulalio, Mano et al. 2012) and are key regulators of heart
remodelling, representing a new class of therapeutic targets in heart failure patients (Philippen, Dirkx et al. 2015).

**Figure 1-4 Schematic Overview of Strategies Available for Heart Regenerative Therapy**

On the top, cell-free therapies are represented by the use of chemical compounds, cytokines, growth factors and microRNAs. At the bottom, cell-based therapies are based on the use of cardiomyocytes derived from different sources such as embryonic stem cells (ESCs), cardiac progenitor cells (CPCs) and induced pluripotent stem cells (iPSCs). Adapted from (Sahara, Santoro et al. 2015).

### 1.5.1 Biological compounds for heart regeneration: microRNAs, as regulators of gene expression

Among all non-coding RNAs, microRNAs (miRNAs) are endogenous 22nt long RNA molecules which act as post-transcriptional regulators of gene expression in plants and animals (Bartel 2004). They exert their function by binding the 3’ UTR of target messenger RNA, leading to either its degradation or the inhibition of protein translation (Humphreys, Westman et al. 2005). MiRNA target recognition is restricted to a short sequence, known as the “seed” sequence (Rhoades, Reinhart et al. 2002, Vasudevan and Steitz 2007) shared by all micro-RNAs belonging to the same family. Interestingly, the length of 3’ UTRs correlates with the number of miRNA-binding sites, therefore with the complexity of gene expression modulation (Osada and Takahashi 2007) (Cheng, Bhardwaj et al. 2009). Moreover, a single miRNA can control the expression of several target mRNAs, therefore enabling the modulation of an entire signaling pathway (van
1.5.1.1 MicroRNA biosynthesis and processing

MicroRNAs have several peculiar features if compared to other functional RNA species in the cell. Most of them are encoded by polycistronic transcripts, suggesting that members of the same family evolved as a cluster (Du and Zamore 2005). In eukaryotic cells, microRNAs are processed in a sequence of steps (reviewed by (Cai, Hagedorn et al. 2004) (Du and Zamore 2005). The precursor microRNA, also known as pre-microRNA, is an approximately 60 nt long hairpin RNA generated by excision from the primary transcript by Drosha (Lee, Ahn et al. 2003) Drosha is a class III endonucleases, which produces duplex RNA products containing a 5’ phosphate and a 3’-OH, with usually a 2 nt overhang at the 3’ end (Zeng, Yi et al. 2005). Drosha exhibits low enzymatic efficiency, therefore a regulatory subunit, DGCR8, helps the enzyme in the recognition of its targets (Fig.1-5).

**Figure 1-5 Biogenesis of microRNAs, processing and maturation.**

A primary miRNA transcript is produced by RNA polymerase II or III and is cleaved by the Drosha–DGCR8 complex in the nucleus. Exportin-5–Ran-GTP mediates the export of the pre-miRNA to the cytoplasm, where Dicer, in complex with TRBP, further processes it to a mature miRNA. The functional strand is then loaded with Argonaute (Ago2) into the RNA-induced silencing complex (miRISC). RISC is guided to silence target mRNAs and to induce mRNA cleavage or translational repression. miRNA function can be artificially mimicked using double-stranded miRNA mimics or inhibited by single-stranded antimiR oligonucleotides, as illustrated in the green box. Adapted from: (van Rooij and Kauppinen 2014).
Once pre-miRNAs are produced, exportin5 (Exp5) is responsible for their export into the cytoplasm (Khvorova, Reynolds et al. 2003), where Dicer, a RNaseIII-type enzyme, recognizes the 3' overhang via its PAZ domain and produces a duplex intermediate miRNA; usually only one of the two strands can be detected in cells, while the other is degraded. How the choice is mechanistically achieved is not known, although it likely involves differential binding and retention of one of the two RNA strands by Dicer and its associated proteins (Tomari, Matranga et al. 2004). The functional strand is bound by Argonaute and loaded into the RNA-induced silencing complex (RISC) (Meister, Landthaler et al. 2004). RISC is guided to silence target miRNAs and to induce mRNA cleavage or translational repression (Fig.1-5).

1.5.1.2 miRNAs controlling cardiomyocyte proliferation
Several microRNAs have been described as powerful regulators of different biological processes. During zebrafish heart regeneration, both mir-99 and mir-100 are downregulated (Aguirre, Montserrat et al. 2014). Interestingly, they both target Smarca5 and Fntb, key factors involved in dedifferentiation and proliferation of adult cardiomyocytes, suggesting that miR-99 and 100 are involved in cardiac cell cycle withdrawal. Other examples are the miR-1 and miR-133 families, part of the same bi-cistronic transcript and both regulators of cardiac cell proliferation. Actually, miR-1 and miR-133a are specifically expressed in cardiac and skeletal muscle, while miR-133b is expressed in skeletal muscle only. Multiple evidence strongly supports a fundamental role for this miRNA cluster during heart development(Zhao, Samal et al. 2005). In fact, Hand2, a crucial transcription factor in cardiac morphogenesis, is a direct miR-1 target. Of note, when an altered expression of miR-1 and miR-133 occurs in adult cardiac tissue, heart failure arises both in mice and humans. This finding can be explained by the fact that miR-133 silencing results in the suppression of genes as Cdc42 and RhoA, which induce the cardiac hypertrophic phenotype (Care, Cataluucci et al. 2007). Similar function is exerted by miR-1, whose overexpression decreases cell death and fibrotic cardiac remodeling (Karakikes, Chaanine et al. 2013). MiR-133-1 and miR-133-2 are inhibitors of cardiomyocyte proliferation, since they both target Cyclin D1 and SRF. MiR-133 also regulates the expression of Mps1, a kinase involved in several cell cycle checkpoints, as the mitotic spindle-assembly checkpoint (Mattison, Stumpff et al. 2011).

The miR-15 family is also involved in cardiomyocyte cell cycle withdrawal. MiR-195 targets Chek1, a checkpoint kinase known to negatively regulate cell cycle progression. As a consequence, miR-15 suppression boosts cardiac cell proliferation and improves cardiac function after MI (Porrello, Mahmoud et al. 2013).
Other microRNAs exert their function in cardiac myocyte differentiation during embryonic heart development, as is the case of the miR-17-92 cluster. The expression of this cluster is under BMP regulation and its direct targets are Isl1 and Tbx1. In mice, their overexpression is sufficient to promote cell cycle entry of cardiac cells and tissue protection in case of MI (Chen, Huang et al. 2013).
Taken together, these findings support the use of miRNA as therapeutic tools to target multiple genes at the same time, in light of the final goal to force cardiomyocyte cell cycle re-entry. In this context, a high-throughput screening performed in our laboratory revealed several human miRNAs able to induce cardiomyocyte proliferation. Among them, hsa-miR 199a-3p and hsa-miR 590-3p were able to induce cardiac regeneration after myocardial infarction, by boosting cardiomyocyte proliferation in vivo (Eulalio, Mano et al. 2012).

1.5.1.3 MicroRNAs in heart regeneration: the role of YAP

The Morrisey’s group (Tian, Liu et al. 2015) recently described the miR-302~367 cluster, which is early expressed during cardiac development in mice and plays an important role in cardiac myocyte proliferation during heart embryogenesis. Main targets of this cluster of miRNAs are several proteins of the Hippo pathway. The sustained expression of miR-302~367 in a transgenic mouse model induced long lasting cardiomyocyte proliferation, finally leading to cardiomegaly and heart failure. Conversely, transient in vivo overexpression of this cluster using miR-302~367 mimics induced heart regeneration, with a significant improvement of cardiac function (Tian, Liu et al. 2015). These observations further support the role of the Hippo pathway as a master regulator of cardiomyocyte proliferation and heart regeneration and validate the use of miRNA mimics as tools to reproduce the effects of specific miRNAs in vivo in a limited time frame, avoiding the adverse effects of their prolonged overexpression.

1.5.2 Chemical compounds for heart regeneration

Parallel to the significant advances in the understanding of key regulatory processes leading to cardiac regeneration, an increasing interest is focused on the development of new, small molecules to be used as therapeutic tools in heart regenerative medicine. Small chemical compounds would significantly improve the impact of heart therapy, with a net reduction in costs and increase of benefits, since already approved drugs have been tested for safety and pharmacokinetics in patients, and they have established manufacturing and distribution networks. To date, only a few chemical compounds have been studied in order to stimulate heart muscle repair. The isoxazole family (Isx) was described to promote cardiac repair after myocardial infarction, activating Notch signaling in progenitors of epicardial origin. Moreover, these organic compounds were able to protect cardiomyocytes after myocardial infarction by inhibiting the proton sensing GPCR-68, a highly controlled receptor during cardiac ischemia. These preliminary encouraging results were not paralleled by a rescue in cardiac function, suggesting a role of Isx in the protection from cardiac remodeling after infarction, rather than in the acute phase of the ischemic damage (Russell, Goetsch et al. 2012).

Another pathway deeply studied as a potential therapeutic target for heart regeneration is the WNT/β-catenin pathway because of its involvement in the cell cycle regulation in the embryonic cardiac precursors (Ueno, Weidinger et al. 2007). ICG-001 is a small WNT modulator molecule, which selectively disrupts the interaction of β-catenin with CREB binding protein (CBP)
(Willems, Spiering et al. 2011). However, ICG-001 ability of improving the cardiac contractile function in an *in vivo* model of myocardial infarction is mainly associated with its effect on epicardial cells rather than on ventricular cardiomyocytes (Sasaki, Hwang et al. 2013).

More recently, MSI-1436, a strong allosteric inhibitor of the protein tyrosine phosphatase 1B (PTP1b), was also described as a promising candidate for heart regeneration. This small molecule, also known as Trodusquemine, showed good tolerance in patients in phase 1 clinical trials for type-2 diabetes and for the treatment of obesity (*ClinicalTrials.gov* identifier NCT number: NCT00606112). Besides these applications, MSI-1436 is able to successfully induce cardiac muscle regeneration in a mouse model of myocardial infarction, with no detectable side effects at a dosage well below the minimum dose tolerated by patients (Smith, Maguire-Nguyen et al. 2017). The regenerative effect of this compound was also observed during regeneration of other tissues, as the caudal fin of zebrafish. This evidence suggests that MSI-1436 could act as a powerful trigger of tissue regeneration in a broad spectrum of putative targets (Smith, Maguire-Nguyen et al. 2017).

Last, but not least, harmine is a compound showing a regenerative effect on pancreatic beta cells; it is a FDA-registered compound, which shares with MSI-1436 the peculiar property to act on many tissues with relevant beneficial effects as discussed below in details.

### 1.5.2.1 Harmine: a versatile compound

The 7-methoxy-1-methyl-9H-pyrido[3, 4-b] indole, also known as harmine, is a tricyclic beta-carboline alkaloid, found in many plants. It was originally extracted from the seeds and root of *Peganum harmala*, a plant broadly used in North Africa, Middle East and Central Asia. It is also present in the stem of the *Banisteriopsis Caapi* vine, the main component of the ayahuasca, a hallucinogen infusion prepared in Brazilian Amazon during tribal spiritual ceremonies.

Harmine is not only present in the vegetable kingdom but also in marine organisms, insects and mammals, as well as in human tissues and fluids. It was traditionally used as a drug with a broad spectrum of features, including antimicrobial, anti-inflammatory, antimycotic, anti-oxidative, antitumor, anti-depressive and analgesic effects (Moloudizargari, Mikaili et al. 2013) (Patel, Gadewar et al. 2012).

### 1.5.2.3 Harmine in biology and its potential therapeutic value

The molecular mechanism of action of harmine is only partially described. It is known that this drug binds type A receptors of the gamma-aminobutyric acid (GABA) in the same site used by benzodiazepine, but with an opposite effect. These chemicals are also known as MAO Inhibitors, due to their ability to inhibit a metabolic enzyme, the monoamine oxidase (MAO), in order to prevent direct activation of monoamine receptors (Farzin, Haghparast et al. 2011).

MAO is responsible for the oxidative deamination of amines and is directly involved in the metabolism of vasoactive and neuroactive amines in many tissues. MAO exists in two different isoforms: MAO-A and MAO-B. Both isoenzymes are able to oxidize dopamine, but MAO-A preferentially acts on serotonin and norepinephrine, while MAO-B on phenylethylamine (Shih,
Chen et al. 1999). In particular, harmala alkaloids reversibly bind the enzymatic site of MAO, resulting in an enhanced and prolonged effect of neurotransmitters, due to the fact that MAO physiological function is to degrade endogenous neurotransmitters and dietary amines. Indeed, at the periphery, MAOs catalyze the oxidative catabolism of circulating amines, blocking the effect of dietary amines. In the central nervous system, MAOs inactivate amine neurotransmitters, guaranteeing the protection of neurons from exogenous amines and monitoring the intracellular reservoir of amines. Genetic deletion of MAO genes results in higher reactivity in stress conditions (Grimsby, Toth et al. 1997). Interestingly, MAO-A-deficient animals show cognitive deficit and reduced control of impulses with behavior disturbances, while a deficiency of MAO-B is not associated with any evident clinical defect, indicating that MAO-A has a more relevant role in amine metabolism (Lenders, Eisenhofer et al. 1996).

Harmine is also a potent inhibitor of a dual specificity tyrosine-phosphorylation-regulated kinase (Dyrk1a), which regulates cell proliferation, apoptosis and brain development (Matsuki, Hori et al. 2005). Dyrk1a belongs to a family of kinases (Dyrk1b, Dyrk2, Dyrk3, Dyrk4a and Dyrk4b), which share the same activation mechanism (Walte, Ruben et al. 2013); they undergo auto-phosphorylation on residue Y321 in order to achieve full activation and phosphorylate their substrates on serines and threonines (Himpel, Panzer et al. 2001). Dyrk1a is also overexpressed in several cancers, such as glioblastoma and lung cancer (Pozo, Zahonero et al. 2013) (Gao, Zheng et al. 2009). A complete overview of Dyrk1a’s downstream molecular mechanism remains a topic of debate.

1.5.2.3.1 Cardiovascular responses

Harmala compounds have a strong impact on the cardiovascular apparatus: bradycardia, alterations in arterial blood pressure and in myocardial contractile force are just a few of several effects on heart and blood vessels.

The vasorelaxant effect induced by harmala drugs is due to their effect on the alpha 1-adrenergic receptors of the vascular smooth muscle cells, and to the increased NO levels, produced by endothelial cells because of the increased levels of external Ca2+. In fact, both harmine and harmaline (10^{-6} to 3x10^{-4} M) are able to inhibit muscle contractile response in a concentration-dependent manner in the presence of stimulatory agents (Berrougui, Martin-Cordero et al. 2006). However, neither harmine nor harmaline showed any effect on the endothelium-independent relaxation.

Interestingly, harmaline and harmine are able to reduce free radicals generated by DPPH, suggesting that the decrease in oxidative stress correlates with an increase of the relaxation effect on aortic rings. Indeed, harmine as well as harmaline are able to reduce the arterial pressure systemically and vascular resistance in peripheral tissues (Berrougui, Martin-Cordero et al. 2006). Little is known about the direct effect of harmine on the myocardium. Pioneering studies described a ionotropic effect and induced reduction in cardiac contractility (Aarons, Rossi et al. 1977). Interestingly, the short time frame of cardiovascular effects is not compatible with the
timing of MAO inhibition by harmala alkaloids, therefore suggesting a MAO-independent mechanism of action (Aarons, Rossi et al. 1977). Another interesting feature of harmine is its strong anti-angiogenic action in tumors both in vivo and in vitro, in a model of gastric cancer, through the downregulation of COX-2 and NO synthase (Hamsa and Kuttan 2010). Harmine is able to inhibit endothelial cell proliferation and induce the production of pro-angiogenic cytochines (VEGF, NO and cytokines such as IL-1b and TNF-a) in a dose-dependent manner. These anti-angiogenic effects were detected at micromolar concentrations (2 µM), supporting the potential therapeutic use of harmine at low dosage.

### 1.5.2.3.2 Nervous System responses

Traditionally, harmala alkaloids have been described to have psychoactive properties with a broad spectrum of effects on both the central and peripheral nervous system. In addition to their effect on MAO inhibition, described above, another important feature of these compounds is related to their anti-depressant effect. Indeed, the acute administration of harmine at 15 mg/kg body weight, in rat hippocampal neurons induced a massive secretion of brain-derived neurotrophic factor (BDNF) (Fortunato, Reus et al. 2009), the levels of which are dramatically reduced in depressed patients.

In the beginning of 1920s, multiple findings brought to evidence the involvement of beta-carboline in Parkinson’s disease development. In particular, patients affected by Parkinson’s disease showed increased levels of β-carbolines with dopaminergic cytotoxicity mediated by DAT (dopaminergic active transporter) (Storch, Hwang et al. 2004). Another neurodegenerative disorder, Alzheimer’s disease, has recently been linked to both MAO activation and Dyrk1a kinase activity. In fact, Dyrk1a contributes to Tau phosphorylation (on Ser396) and consequently to the formation of the neurotoxic tau aggregates (Frost, Meechoovet et al. 2011), responsible for the pathological progression of Alzheimer’s disease. In this context, harmine treatment was shown to inhibit the formation of these aggregates, due to its suppressing activity on MAO and Dyrk1a (Frost, Meechoovet et al. 2011).

### 1.5.2.3.3 Anti-cancer properties

In Iran and Morocco, traditional medicine preparations for the treatment of many tumors were based on *P. harmala* seeds. These habits raised the interest of many researchers to understanding the properties of these seeds and, specifically, of β-carboline alkaloid compounds, which are supposed to be partially responsible for the inhibition of cancer cell proliferation. Additionally, their cytotoxic action was also linked to the ability to interact with nucleic acids, therefore inhibiting DNA synthesis (Chen, Chao et al. 2005). Actually, in vitro, harmine shows a very strong effect on the inhibition of DNA topoisomerase I purified from human placenta (Sobhani, Ebrahimi et al. 2002). Moreover, harmine mediates DNA conformational changes that interfere with DNA synthesis, resulting in proliferation arrest and genotoxic effect in budding yeast (Li, Liang et al. 2007).

To further strengthen the role of harmine in cell-cycle related processes, recent findings have
shown that, in colon cancer cells, harmine decreases cyclin D1 expression levels, with an opposite increase of cyclin A, E2 and B1 levels as well as of the levels of CDK1/cdc2, myt-1 and p-cdc2. This expression profile is a hallmark of an S-G2/M phase arrest. Moreover, in this model, harmine led to activation of both caspase 3 and PARP and the downregulation of Bcl-2 and Mcl-1; all these events are typically associated with apoptotic death mediated by mitochondrial pathway activation and by inhibition of both Akt and ERK signaling (Liu, Li et al. 2016). In hepatic cancer cell lines, harmine was shown to inhibit Rad51 recruitment, affecting homologous recombination and inhibiting double-strand breaks repair, with consequent cytotoxicity (Zhang, Zhang et al. 2015). This work identified novel potential targets in the homologous recombination pathway to optimize cancer therapy and arrest cancer cell proliferation. Of note, it seems that harmine is able to specifically target proliferating cells, as in hepatoma cell lines, with minimal side effect on post-mitotic cells.

Another example of tumor cells sensitive to harmine treatment is head and neck squamous cell carcinoma (HNSCC). In this model, harmine-mediated inhibition of Dyrk1a decreases phosphorylation of the transcription factor Foxo3a, thus promoting cell death (Radhakrishnan, Nanjappa et al. 2016). The aforementioned examples are just a few of the several ones supporting harmine as an anticancer drug. Its cytotoxic effect is further strengthened by its anti-angiogenic and anti-inflammatory properties (discussed above), which supported the patent on harmine-anti-tumor effect registered in 2001 (code: CN1358720A).

1.5.2.3.4 Osteogenic effects

The increasing need of new drugs to treat bone-destructive diseases led to the investigation of the properties of natural compounds in bone reabsorption. In this setting, harmine acts on osteoclast progenitors but not on osteoblasts, inhibiting osteoclastogenesis. In particular, harmine downregulates c-Fos and Nfatc1 expression, targeting nuclear factor kappa-B ligand (RANKL) signaling pathway and preventing osteoclast precursor differentiation (Yonezawa, Hasegawa et al. 2011). As a net result, harmine acts on osteoblast differentiation stimulating the activation of Runx2 and BMPs signaling. As in the case of its anti-tumoral effect, a patent for the use of alkylamine harmine derivatives to promote bone repair was registered in 2014 (code: EP2968284A2).

1.5.2.3.5 Anti-diabetic actions

Among the alkaloids present in P. harmala, harmine is the main responsible of its anti-diabetic effects. Interesting findings show that this compound has an important metabolic effect, promoting the specific expression of PPARγ in adipocytes, resulting in an improvement of glucose metabolism (Waki, Park et al. 2007). Furthermore, harmine is also able to attenuate the expression of pro-inflammatory genes and diminishes macrophage recruitment in the adipose tissue, which is linked to the insulin resistance induced by obesity. The action of harmine on adipocyte metabolism can partially be explained by the inhibition of WNT signaling pathway (Waki, Park et al. 2007).
Since the deficiency of functional pancreatic beta-cells represents the cause of type 1 diabetes, harmine represents a promising therapeutic agent, as it is able to promote beta-cell proliferation, increase islet mass and improve glucose metabolism in *in vivo* models. The inhibition of Dyrk1a and the NFAT transcription factors were reported to be responsible for the harmine pro-proliferative effect in these settings (Wang, Alvarez-Perez et al. 2015).
2 Thesis Aim

The work presented in this Thesis was aimed at identifying the molecular mechanisms by which a few microRNAs and one small molecule are capable to stimulate cardiomyocyte proliferation ex vivo and cardiac regeneration after myocardial infarction in vivo.

First, I concentrated my attention on the effect of 10 microRNAs that had been previously identified by the laboratory for their capacity to stimulate proliferation of neonatal rodent cardiomyocytes. For 6 of these microRNAs, I determined the transcriptional profile induced by their transfection into cardiomyocytes, observing that they all impinge on the Hippo/YAP pathway and determine remodelling of the actin cytoskeleton by targeting one or more of the proteins that control actin polymerization.

Next, I screened a library of FDA-registered drugs in search for small molecules able to activate cardiomyocyte proliferation. I discovered that the neuroactive alkaloid harmine exerted a powerful pro-proliferative effect, which was mediated by the inhibition of the cellular Dyrk1a kinase. Similar to the investigated microRNAs, activation of YAP was also essential to mediate the pro-proliferative effect of harmine.

These findings are discussed in the context of the possibility of inducing cardiac regeneration after myocardial infarction by the stimulation of the endogenous capacity of cardiomyocytes to proliferate.
3 Materials and Methods
3.1. Cell culture methods

3.1.1 Cell lines: HeLa
HeLa cells (ATCC® Cells were taken in a Dulbecco’s modified Eagle medium with 1 g/l glucose (DMEM, Life Technologies) supplemented with 10% FBS and 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma).

3.1.2 Primary cells: isolation of ventricular cardiac myocytes from neonatal rats
Ventricular CMs were isolated from neonatal rat as previously described (Collesi, Zentilin et al. 2008). In brief, ventricles from neonatal (P0) rats were separated from the atria, cut into pieces and dissociated in calcium and bicarbonate-free Hanks with Hepes (CBFHH), using mechanical and physical stirring. CBFHH buffer contains 1.75 mg/ml trypsin (BD Difco) and 10 mg/ml DNase II (Sigma). Tissue digestion was performed at room temperature in ten minute steps, with collection of the supernatant to fetal bovine serum (FBS, Life Technologies) after each step. In order to pellet the collected supernatant to fetal bovine serum (FBS, Life Technologies) after each step. In order to pellet cells, collected supernatant was centrifuged and cardiac cells were then resuspended in Dulbecco’s modified Eagle medium 4.5 g/l glucose (DMEM, Life Technologies) supplemented with 5% FBS, 20 mg/ml vitamin B12 (Sigma) and 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma). The collected cells were filtered using a cell strainer (40 mm, BD Falcon) and finally seeded onto four 100-mm plastic dishes for 2 hr at 37°C in 5% CO2 and humidified atmosphere. CMs in the supernatant were then collected, counted and plated at the appropriate density into BD Matrigel Matrix Growth Factor Reduced (356230 BD Bioscience) pre-coated Primaria 96-well plates (BD Falcon) or matrigel-coated Primaria 35-mm dishes (BD Falcon).

Number of cardiac cells seed for each plate/dish:
96-multiwell plate: 15*10^3 cells/ well
35 cm dishes: 5*10^5 cells/ dish
60 cm dishes: 1*10^6 cells/ dish

3.1.3 microRNA/siRNA transfection and EdU incorporation
MicroRNAs and siRNAs (SMARTpool) were obtained from Dharmacon, Thermo Scientific and transfected into neonatal rat CMs using a standard reverse transfection protocol (Eulalio, Mano et al. 2012). Cardiomyocyte transfection efficiency with miRNAs/siRNAs is >90% using Lipofectamine RNAiMAX as lipids. In brief, transfection lipids (Lipofectamine RNAiMAX, Life Technologies) were incubated with OPTIMEM (Life Technologies) for 5 min at RT and added to miRNAs, siRNAs or a combination of the two (at a final concentration of 25 nM each nucleic acid), arrayed on 96-well plates; 30 min later, 1x10^4 cells were seeded in each well. Twenty-four hours after transfection, culture medium was replaced by fresh medium; 28 h after
plating), the culture medium was supplemented with 5 mM 5-ethynyl-29-deoxyuridine (EdU, Life Technologies) for 20 h. Cells were fixed at 72 h after plating and processed for immunofluorescence. Experiments were performed in quadruplicate.

**Oligonucleotides**

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<td>siRNA NT4</td>
<td>Ambion</td>
</tr>
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</table>

3.1.4 Chemicals

Harmine, harmaline, harmane (Sigma-Aldrich) were dissolved in DMSO and further diluted in culture media at the final concentration of 10 µM.

CytochalasinD (sc_201442, Santa Cruz) was added to CM culture medium at the final concentration of 0.1 µM.

3.1.5 TEAD reporter assay

For luciferase assays, CMs were plated at the concentration of 1.5x10^5 (day 0) in a double-matrigel coated 96-Multiwell plate and, the day after, were transfected with the selected miRNAs (Dharmacon). At day 3 of culture, cells were transfected with 100 ng of both synthetic TEAD reporter firefly luciferase plasmid (p8xGTIIC-Lux, Plasmid #34615, Addgene) and a renilla luciferase plasmid. The day after transfection, firefly and renilla luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega), according to the manufacturer’s instructions.

3.1.6 Luciferase 3′-UTR reporter assays

Constructs coding for rat 3′-UTR of Cfl2, TAOK1, β-TrCP or STK38L (gBlock) were obtained by gene synthesis from Biomatik (3′UTR-Cfl2) or Genewiz (3′UTR- β- TrCP and 3′UTR-TAOK1) and subcloned into psiCHECK2.1 vector (Promega). HeLa cells were transfected with 100 ng of
the reporter constructs or psiCHECK2 vector (control) using FuGENE HD transfection reagent (Promega). Twenty-four hours later, cells were transfected with the miRNA under investigation at a final concentration of 50 nM in 96-well plates, using a standard forward transfection protocol similar to that described above. Each condition was tested in quadruplicates.

Firefly and renilla luciferase activities were measured 48 hr after plasmid transfection using the Dual Luciferase Reporter Assay System (Promega), according to the manufacturer’s instructions.

### 3.1.61 mutated UTR-luciferase reporters: construct generation

The mutated 3’-UTR of β-TrC and TAOK1 were obtained by gene synthesis (gBlocks) from IDT (Integrated DNA Technology) and sub-cloned into the psiCHECK2.1 vector. The gBlocks terminal regions were implemented with enzymatic restriction sites for XhoI and NotI in addition to flanking regions of stuffer-DNA to increase cut efficiency.

The gBlocks were digested with XhoI and Not-HF (New England Biolabs) as well as the wt UTR-luciferase reporter plasmids.

Briefly, 500 ng of gBlock or 2µg of the vectors were digested with 0.1µl/0.5µl of XhoI (10000U/ml) and 0.1µl/0.5µl of Notl-HF (10000U/ml) in presence of 2µl/3µl of 10x Buffer 2.1 and H2O to final volume of 20 µl/30µl and incubated at 37° for 2 hours; then, heat inactivation for 15’ at 65°C.

Both digested vectors were submitted into DNA gel electrophoresis. 1% agarose gel was prepared, placed into an electrophoresis chamber and rinsed in TBE (Tris-Borate-EDTA buffer; Sigma). Samples were loaded into pre-cast wells and placed in an electric field where due to the negatively uniform mass/charge ratio of nucleic acids, DNA fragments migrated according to their molecular weight and secondary structure. The linearized vectors were excised and purified from gel using Wizard SV Gel and PCR Clean-Up System (Promega) according to manufacturer’s instruction. Also the digested gBlocks were purified, using the same kit. Finally, the linearized vectors and the digested gBlocks (mutated 3’UTR of β-TrC and TAOK1, respectively) were ligated and transformed into XL10 Gold (Stratagene) recombination deficient bacteria. Colonies were screened for ampicillin resistance as well as insert size by restriction enzyme digestion.

Before using the ligation products to perform the 3’UTR-luciferase assay, we checked the sequence of these plasmids by sequencing (GATC-biotech).
**Figure 3-1 Schematic representation of the 3’UTR of TAOK1 and β-TrC transcripts**

The 3’UTR of TAOK1 and bTrC mRNA were mutated as shown in the miR-199a-3p recognized region (in blue). In green the seed sequence of miR-199a-3p.

### Recombinant DNA

<table>
<thead>
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<td>Gift from S. Piccolo Lab, Dupont et al. Nature 2011</td>
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<td>3’UTR-Stk38L plasmid</td>
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</table>

### 3.1.7 High-throughput screening: FDA-registered compound screening

The miRNA mimics corresponding to hsa-miR-590-3p, hsa-miR-199a-3p were obtained from Dharmacon, Thermo Scientific. For miRNA transfection in rat CMs, miRNAs were robotically transferred from stock library plates to Primaria 384-well plates (BD Falcon) leaving columns 1 and 24 empty for addition of controls (buffer, cel-miR-67). MiRNAs were transfected into neonatal rat CMs using a standard reverse transfection protocol, at a final miRNA concentration of 25 nM. Briefly, the transfection reagent (Lipofectamine RNAiMAX, Life Technologies) was diluted in OPTI-MEM (Life Technologies) and added to miRNAs on the 384-well plates; 30 min later, 5,000
cells/well were seeded. Twenty-four hours after transfection, culture medium was replaced by fresh medium; 24 h later, that is, 48 h after plating, FDA-registered compounds, 10 mM (or DMSO as control) were robotically arrayed on the 384-well plates and 52h after plating 5 µM 5-ethynyl-2’-deoxyuridine (EdU, Life Technologies) was added for 20 h. Cells were fixed at 48 h after plating and processed for immunofluorescence. Screening was performed in duplicate. Cells were stained according to IF standard protocol as described later.

3.1.8 Immuno-staining: immunofluorescence on primary cell cultures
CMs were fixed with 4% PFA for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 10 min, followed by 1 hr blocking in 2% BSA (Roche). CMs were then stained overnight at 4°C with mouse monoclonal antibody against sarcomeric α-actinin (Abcam) diluted in blocking solution. Cells were washed with PBS and incubated for 2 hr with the secondary antibody conjugated to Alexa Fluor-488 (LifeTechnologies). Cells were further processed using the Click-IT EdU 555 Imaging kit (Life Technologies) to reveal EdU incorporation, according to the manufacturer’s instructions, and stained with Hoechst 33342.

3.1.8.1 Image acquisition
In screening experiments, image acquisition was performed using an ImageXpress Micro automated high-content screening fluorescence microscope at ×10 magnification; a total of 16 images were acquired per wavelength, well and replicate, corresponding to approximately 2.500 cells analyzed per condition and replicate. Image analysis was performed using the ‘Multi-Wavelength Cell Scoring’ application module implemented in MetaXpress software (Molecular Devices). Cells were scored as proliferating only if positive for the proliferation marker EdU. In all quantifications, CMs were distinguished from other cells present in the primary cultures by their positivity for sarcomeric α-actinin.

3.2 Biochemistry

3.2.1 Nuclear/cytoplasmic fractionation
1x10^6 neonatal rat cardiac myocytes were plated on matrigel-coated Primaria 60-mm dishes (BD Falcon) as described above. miRNAs were transfected by a standard forward transfection protocol. Seventy-two hr after transfection, cells were washed with ice-cold PBS and harvested in 80 µl of hypotonic buffer (50 mM Tris pH7.5, 10 mM NaCl, 3 mM MgCl2, 10% glycerol) supplemented with protease inhibitors (complete tablets, Mini EDTA-free, Roche) and phosphatase inhibitors (cocktail 3 Sigma, 2mM orthovanadate, 1 mM NaF). After 1 min, cells were scraped and NP40 was added at 0.1% final concentration. After 5 min on ice, cells were centrifuged at 4000 rpm for 5 min at 4°C; the supernatant and cytosolic fractions were then recovered. Pellets were resuspended in 70 µl IPLS buffer (50 mM Tris pH 7.5, 120 mM NaCl, 1 mM EDTA, 0.5% NP40, supplemented with the same inhibitors as above) and sonicated with 3 pulses of 10 min each (30 sec on/30sec off) on
ice in a Bioruptor™ Plus (Biosense). Nuclear lysates were centrifuged at 16,000 rpm for 20 min at 4ºC and the supernatant was recovered as nuclear fraction. Concentration of cytoplasmic and nuclear lysates were measured using the Bradford assay (Protein Assay Dye Reagent Concentrate, Bio Rad). Lysates (10 to 20 µg) were loaded for protein separation and subsequent blotting onto a PVDF membrane (Amersham™ Hybond™ PVDF, GE Healthcare).

### 3.2.2 Antibodies

The following antibodies were used for western blotting analysis.

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### 3.2.3 G/F actin separation and immunoblotting

The amount of G-actin and F-actin in CMs was quantified using G-actin/F-actin in vivo assay kit (Cytoskeleton Inc.) according to the manufacturer's instructions. Briefly, cells were lysed in pre-warmed lysis/F-actin stabilizing buffer supplemented with protease inhibitor and ATP. The cell lysate was centrifuged for 5 min at 350°—g to remove debris. A 100 µl aliquot was then ultracentrifuged at 100,000xg for 1 hr at 37ºC to pellet F-actin, with G-actin remaining in the supernatant. The pellet was resuspended in 100 µl of F-actin destabilizing buffer on ice for 1 hr
with frequent pipetting. Equal volumes of G-actin and F-actin fractions were mixed with 5x loading buffer and run on SDS-PAGE. Western blot analysis was performed using the anti α-actin antibody provided in the kit. Densitometry analysis was performed using the ImageJ software.

### 3.3 Molecular Biology Methods

#### 3.3.1 RNA isolation and quantitative Real-Time PCR

Total mRNA was isolated from CMs 72 hr after transfection, or at different time points after cytochalasin D treatment, using a standard TRIZOL RNA isolation protocol. The RNA obtained (1 µg) was reverse-transcribed using MLV-RT (Invitrogen) with random hexamers (10 µM) in a 20 µl reaction, following the manufacturer's instructions. mRNA levels for CTGF, CyR61, BIRC5 and GAPDH were measured by Real-Time PCR using pre-designed TaqMan assays (Thermofisher) and iQ™Supermix (Bio-Rad) according to the manufacturer’s instruction. TaqMan Probes are listed in the table.

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<th>Real Time-PCR probes</th>
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#### 3.3.2 Transcriptomic analysis

Deep-sequencing of total neonatal rat CM RNA was performed 72 hr after transfection of microRNAs by IGA Technology Services, Udine, Italy as in ref. (Eulalio, Mano et al. 2012). Briefly, RNA purity, integrity and concentration were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies). Only RNA with a RIN value >7 and an rRNA 28S/18S ratio >2 was taken forward for sample preparation. Two µg of total RNA (minimum concentration of 200 ng/µl) per sample were sequenced on an Illumina HiSeq2000. Two lanes in 7-plex were run obtaining 2 millions of single-reads per sample, 50-bp long. The raw sequencing data discussed in this thesis have been deposited in NCBI's Sequence Read Archive (SRA) and are accessible
through SRA STUDY accession SUB2866022.

Real-time image analysis, base calling, de-multiplexing and production of FASTQ sequence files were performed on the HiSeq2000 instrument using the HiSeq Software. Raw sequence files were quality checked using FASTQC software (www.bioinformatics.babraham.ac.uk/projects/fastqc) and trimmed to remove Illumina adaptor using Cutadapt software (Martin, 2011). RNA-seq reads were mapped to Rattus norvegicus reference genome (GCF_000001895.5 Rnor 6.0.82) and to known mature miRNAs using STAR software (Dobin et al., 2013). Rounded Gene counts were used as input and transformed to rpkm using Bioconductor package edgeR rpkm function (McCarthy, Chen et al. 2012). The CLC Bio Genomic Workbench (Mortazavi et al., 2008) was used to quantify gene expression levels. In particular, sequenced RNA fragments were mapped against the NCBI Rnor_6.0 annotation release 106 (GCF_000001895.5) RefGene genome annotation (O'Leary, Wright et al. 2016). The resulting gene expression levels were then normalized in RPKM (reads per kilobase of exon model per million mapped reads). In case of transfection of a pro-proliferative miRNA, genes whose RPKM values were greater than 1.00 in both miRNA and cel-miR-67 control-transfected rat CMs were considered as expressed. Fold changes were taken with respect to the expression of cel-miR-67 control. Genes whose fold changes were greater than 1.3 (both upregulated and downregulated) were considered as differentially expressed.

### 3.3.3 Clustering of fold change expression levels

Cluster analysis was performed on the basis of the log2-fold changes in gene expression in respect to cel-miR-67 to classify the pro-proliferative miRNAs according to their effects on CMs upon transfection. In brief, the correlation between the log2-fold changes for all pairs of miRNA was calculated. Clusters were then hierarchically identified using the average linkage criterion with a Euclidean distance metric as implemented in the clustering package of SciPy v0.18.1 (http://www.scipy.org). Dendrograms were then generated to visualize the arrangement of the resulting cluster.

### 3.3.4 Bioinformatic target prediction

To the best of our knowledge, bioinformatic predictions of seed sequence interactions with rat transcripts are not available. With this constraint, we compiled a list of rat miRNA-gene interactions from mouse predictions. In particular, predicted mouse gene targets of the seed sequences (corresponding to miRNA families) of pro-proliferative miRNAs were collected from TargetScanMouse Release 7.1 (Agarwal, Bell et al. 2015). The mouse genes were then converted to their corresponding rat genes via homology using the HomoloGene database (Coordinators 2016). The list of miRNA-gene interactions was filtered to only include genes that were downregulated by the miRNA upon transfection to CMs according to the transcriptomic data.
3.4 Animal models

Wistar rats and CD1 mice were purchased from Charles River Laboratories Italia Srl. Animal care and treatments were conducted in conformity with institutional guidelines in compliance with national and international laws and policies (EEC Council Directive 86/609, OJL 358, 12 December 1987).

Dyrk1a F/F mice were received by Prof. John D Crispino from the Robert H Lurie Medical Research Center and MHC-MCM-Dyrk1a F/F mice were generated by breeding Dyrk1a F/F mice and MHC-MCM- mice, which are available in ICGEB Animal House Facility.

3.4.1 Myocardial infarction

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 with an 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. 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. EdU was administered intraperitoneally (500 µg per animal) every 2 days, for a period of ten days.

3.4.2 Echocardiographical analyses

To evaluate left ventricular function and dimensions, transthoracic two-dimensional echocardiography was performed on mice sedated with 5% isoflurane at 12, 30 and 60 days after myocardial infarction, using a Visual Sonics Vevo 2400 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. Echocardiography analysis was performed at week 1, 2, 4 and 8 after infarction (in harmine-treated animals) or after the induction of the phenotype (Dyrk1a KO mice) and hearts were collected at 8 week after infarction (n= 6 animals per group).

3.4.3 Heart collection and histological analysis

At the end of the studies, animals were anaesthetized with 5% isoflurane and then killed by injection of 10% KCl, to stop the heart in diastolic phase. The heart was excised, briefly washed in PBS, weighted, fixed in 10% formalin at room temperature, embedded in paraffin and further processed for histology or immunofluorescence. PicusSirius-Red staining were performed according to standard procedures and analyzed for morphology and extent of fibrosis.
3.5 Statistical analysis

Unless otherwise indicated, all data are expressed as mean ± standard error of the mean (SEM). One-way ANOVA followed by post-hoc analysis with the Bonferroni’s Multiple Comparison Test was used for the analysis of multiple groups; two-tail Student’s t-test was used to compare two individual groups.
4 Results
4.1 Activation of the YAP transcriptional coactivator mediates function of miRNAs inducing cardiomyocyte proliferation

We wanted to explore the involvement of the YAP-Hippo signaling pathway in the biological effect of a series of human microRNAs previously identified to promote cardiac cell proliferation (Eulalio et al 2012). To this aim, we evaluate TEAD activation rate by transfecting neonatal rat cardiomyocytes with a TEAD-responsive reporter plasmid (p8xGTII-Lux) together with the top 10 of microRNAs characterized in our previous work for their pro-proliferative ability (Fig. 4-1A). These included human miR-590-3p (the most effective in our original screening in rat cells), miR-199a-3p (the most effective in mouse cells), miR-1825, miR-302c, miR-302d and miR-373 (two miRNAs known to be enriched in embryonic stem cells (Barroso-del Jesus et al., 2009), miR-33b*, miR-18a*, miR-1248, miR-30e*. C. elegans cel-miR-67 was used as a negative control, while constitutively activated YAP (YAP5SA) was used as a positive control. We found that TEAD-luciferase reporter activity was significantly increased upon treatment with all the selected microRNAs compared to the control cel-miR-67 (Fig. 4-1B).

**Figure 4-1. YAP activation in neonatal rat cardiomyocyte upon treatment with pro-proliferative microRNAs**

A. Experimental scheme to test activation of TEAD-dependent transcription. B. TEAD luciferase reporter analysis of CMs transfected with the indicated miRNA mimics. Transfection efficiency was standardized over a constitutively expressed Renilla luciferase reporter. Transfection of a constitutively activated YAP plasmid (pYAP5SA) was used as a
positive control. Data are mean ±SEM (n=5 independent experiments); *P<0.05, **P<0.01; one-way ANOVA C. Increase in YAP nuclear level by CM treatment with proproliferative miRNAs. Western blot of a representative experiment show the levels of nuclear and cytoplasmic YAP1 and phospho-YAP1 (P-YAP1) 72hr after transfection. PARP1 and GAPDH were used for loading control of the nuclear and cytoplasmic fractions respectively. D. Quantification of the increase in YAP nuclear level in CMs after miRNA mimics transfection. Results are shown as a ratio of YAP to nuclear PARP1. Data are mean ±SEM (n=5 independent experiments); *P<0.05, **P<0.01; one-way ANOVA.

To verify that detected TEAD activation corresponded to an increase of nuclear YAP, we investigated YAP1 levels in both cytoplasmic and nuclear fractions of cardiac cells treated with the 10 pro-proliferative microRNAs. In line with the previous evidence, we found that, in all cases, there was a correlation between cell treatment with the pro-proliferative microRNAs and the increase in YAP1 levels in the nucleus as shown in Fig. 4-1C and 4-1D. Finally, to understand whether YAP activation was a crucial event for CM proliferation mediated by these miRNAs, we knocked down YAP1 using a specific siRNA (YAP1 knock down >85% is shown in Fig. 4-2A) and simultaneously treated CMs with the investigated miRNAs. We analyzed EdU incorporation level as a marker for cell proliferation and we found that YAP1 knockdown prevented the pro-proliferative effect of all the microRNAs under investigation, as shown in Fig. 4-2B. Representative images are shown in Fig. 4-2C.

From these results, we conclude that the increase in nuclear YAP activation is a common key event, which mediates CM proliferation by the ten microRNAs under investigation.
**Figure 4-2. YAP is crucial to sustain cardiomyocyte pro-proliferative effect exerted by the microRNAs under investigation**

A. Efficacy of YAP1 downregulation using a specific siRNA. On the left side, representative western blotting. On the right side, quantification of 3 independent experiments. Data are mean ± SEM; **P<0.01; t-test.

B. Induction of CM proliferation by miRNA mimics is blunted by YAP knockdown. CMs were transfected with pro-proliferative microRNAs alone or in combination with an anti-YAP siRNA. The graph shows the percentage of sarcomeric α-actinin-positive cells that incorporated EdU. Data are mean ± SEM (n=4 independent experiments); *P<0.05, **P<0.01; one-way ANOVA.

C. Representative images of CMs treated with the indicated miRNAs with or without a siRNA against YAP.
visualized in green as sarcomere α-actinin-positive cells. Hoechst staining was used for nuclei (blue). EdU incorporation is visualized in red (bottom panels). The arrows indicate example of proliferating CMs. Scale bar: 100 µM.

4.2. Common regulatory pathways mediate the activity of pro-proliferative miRNAs

Next, to understand the mechanisms used by the microRNAs of interest to obtain YAP activation, we first analyzed the transcriptional feature of neonatal rat cardiomyocytes treated with six of these microRNAs, namely human miR-590-3p, miR-199a-3p, miR-1825, miR-302d, miR-373 and miR-33b*; C. elegans cel-miR-67 was used as a negative control. RNA from miRNA-transfected cardiac myocytes was isolated and used for transcriptomic RNA sequencing followed by computational analysis (Fig. 4-4A and -4B). Cluster analysis was performed on the obtained results, showing that miR-302d and miR-373-3p had similar effects on target gene regulation (as shown in Fig. 4-4C), as predictable from their common seed sequence (Fig. 4-3).

<table>
<thead>
<tr>
<th>MirBase accession</th>
<th>MirBase ID</th>
<th>sequence</th>
<th>Genome context</th>
</tr>
</thead>
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<td>MIMAT0000232</td>
<td>hsa-miR-199a-3p</td>
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<td>chr17: 17813858-17813916 [-].</td>
</tr>
<tr>
<td>MIMAT000693</td>
<td>hsa-miR-30e-3p</td>
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<td>chr3: 41220027-41220118 [+].</td>
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<td>5’ GAUGAGUCUCGAUUUGUGGUUGGUU 3’</td>
<td>chr19: 53788710-53788770 [+].</td>
</tr>
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</table>

FIGURE 4.3. miRNA SEQUENCE AND CHROMOSOMAL LOCALIZATION

Sequence, miRBase accession number, miRBase ID and chromosomal localization of the human miRNAs under investigation. The miRNA seed sequence is in red.

Fig. 4-4A schematically shows the number of genes from NCBI database that were differentially regulated by the miRNAs: a total of 3734 genes were downregulated by at least 1 miRNA (at 1.0 RPKM cutoff and 1.30 fold-change cutoff), while 3326 were upregulated. Next, we performed a pathway analysis using IPA software (Build version: 389077M, Content version: 27821452, 08/08/2016), considering all the tested miRNAs as replicates of the common CM proliferative phenotype. The results showed that the network with the higher score (34) was the cell cycle-cancer and cardiovascular system development and function network, followed by the embryonic-organismal-tissue development network with a score of 22.

Given the involvement of the YAP-Hippo signaling pathway on the regulation of cardiomyocyte
proliferation during embryonic development (Xiao et al., 2016), we focused our attention on genes linked to this pathway. To this aim, we generated an update catalogue of the genes known to have a role in the regulation of YAP-Hippo signaling pathway (203 genes). For each gene we reported fold changes in gene expression for each miRNA treatment along the bioinformatics prediction of miRNA targeting (TargetScan software) (Table 1).

A summary of all this information is reported in Fig. 4-4D; the level of each transcripts is indicated by its fold difference over control (green: downregulated genes; red: upregulated genes). Interestingly, five out of six microRNAs upregulated TEAD2 levels, the final effector of the Hippo pathway. Moreover, all the selected microRNAs targeted either LATS1/2 or STK38L kinases, which blunt CM proliferation via YAP1 inactivation (Hergovich, 2016).

Together, these bioinformatics findings on RNA sequencing data indicate that there is a signature of Hippo pathway involvement upon treatment of cardiomyocytes with the six analyzed pro-proliferative microRNAs.
**Figure 4-4. Expression profile of genes in neonatal CMs upon treatment with pro-proliferative miRNAs**

Total neonatal rat CM RNAs were deep-sequenced and quantified using standard protocols. The gene expression profile mapped on Rattus norvegicus reference genome (GCF_000001895.5 Rnor 6.0.82) was then normalized by RPKM. Fold-changes in gene expression upon transfection of pro-proliferating miRNAs were taken with respect to cel-miR-67 (negative control). **A.** Schematic representation of RNAseq experiment. Neonatal rat CMs were reverse transfected with the indicated miRNAs and RNA was analyzed by Next Generation Sequencing (NGS). **B.** Heatmap showing differentially expressed genes in response to all 6 miRNAs; downregulated genes are in green, upregulated genes in red. **C.** Cluster analysis was performed on the basis of log2 (fold-change over control, cel-miR-67). The dendrogram shows the arrangement upon hierarchically clustering using an average linkage criterion with a Euclidean distance as a metric. **D.** Common regulatory pathways mediate activity of miRNAs inducing CM proliferation, among which Hippo and F-acting polymerization pathways. Upregulated genes are in red, downregulated genes in green.
4.3. Hippo pathway downregulation activates CM proliferation

Interestingly, miR-199a-3p, one of the most effective miRNAs in boosting CM proliferation, lead to the downregulation of three mRNA targets indirectly involved in the Hippo pathway, namely those coding for the TAO kinase1 (TAOK1), the E3 ubiquitin-ligase β-transducing repeat containing protein (β-TrCP) and the serine/threonine kinase, STK38L. TAOK1/3 was described to activate MST1/2 via phosphorylation (Boggiano et al., 2011), as well as LATS1/2 (Plouffe et al., 2016; Poon et al., 2011); β-TrCP mediates YAP ubiquitination and consequently leads to its degradation (Zhao et al., 2010). STK38L, a kinase belonging to the same AGC serine/threonine kinase family as LATS1/2, directly inactivates YAP via phosphorylation (Hergovich, Stegert et al. 2006) (Fig. 4-5A). In order to validate these targets and the direct binding of their 3’UTR by the studied miRNAs, we cloned the 3’UTRs of TAOK1, β-TrCP and STK38L mRNAs downstream the firefly luciferase gene and transfected these plasmids into HeLa cells treated with the 10 pro-proliferative miRNAs (Fig. 4-5B). Co-transfection of a Renilla luciferase plasmid served as a control of transfection, while the cloning plasmid (psiCheck2.1) was used for normalization. Analysis of the luciferase activity 48 hours after transfection revealed that only TAOK1 and β-TrCP, but not STK38L, were 3’UTR-direct targets of miR-199a-3p (Figs. 4-5C, -5D and -5E respectively). The mutated counterparts were no longer able to bind miR-199a-3p as shown in Fig. 4-5F and -5G. In the case of STK38L, we found a downregulation of its transcript upon miR-199a-3p, miR-302d and miR-373 treatment, possibly due either to an indirect effect or to the binding of miRNAs outside the 3’UTR (western blotting analysis and protein quantification are reported in 4-5M and -5N). To complete this functional characterization, the downregulation of both TAOK1 and the β-TrCP by miR-199a-3p transfection was detected both at protein and mRNA levels (Fig. 4-5H, -5I and -5J show representative western blots and quantifications; gene expression levels are reported in Fig. 4-5K, -5L).
**Figure 4-5. Mechanism for YAP activation by miR-199a-3p and other miRNAs**

A. Schematic representation of the Hippo pathway, with the indication of the predicted target proteins according to the TargetScan software. **B.** Experimental flow chart of 3'UTR luciferase assays. Fluc and Rluc: firefly and Renilla luciferase genes, respectively. 3'UTR luciferase assays with the 10 pro-proliferative miRNAs. **C.** TAOK1 and β-TrCP (D) 3'-UTRs are direct targets of miR-199a-3p, while STK38L is not (E). **F-G** Mutated 3'-UTR luciferase assay of both TAOK1 (F) and β-TrCP (G). All the Renilla (Rluc) values were normalized over firefly luciferase (Fluc) values. Control refers to transfection of a firefly luciferase gene with no 3'UTR. **H-J.** Representative western blots showing downregulation of TAOK1 and β-TrCP proteins in cells treated with miR-199a-3p mimic (H) and the relative
quantifications (I-J). K-L. Real-time RT-PCR quantification of TAOK1 and β-TrCP mRNAs in CMs treated with miR-199a-3p mimic. Data are mean ±SEM (n=3 independent experiments); *P<0.05; t-test. M. Western blot showing STK38L levels upon treatment with miR-199a-3p. A siRNA targeting STK38L was used as a negative control. N. Protein quantification of western blot shown in panel M. Data are mean ±SEM (n=3 independent experiments); *P<0.05; t-test. O. RT-PCR quantification of STK38L upon pro-proliferative miRNA transfection. Data are mean ±SEM (n=3 independent experiments); *P<0.05, **P<0.01; one-way ANOVA.

Next we investigated whether the specific silencing of TAOK1, β-TrCP and STK38L had a direct impact on CM proliferation. We co-transfected neonatal CMs with a TEAD luciferase plasmid and with siRNAs targeting TAOK1, β-TrCP or STK38L (Fig.4-6C). SiRNA downregulation of each of these targets was evaluated at the protein level; β-TrCP expression was silenced >70% (Fig. 4-6A); TAOK1 expression >90% (Fig. 4-6B); and STK38L >65% (Fig. 4-5N), compared to control. Silencing of these proteins resulted in the transcriptional response of the TEAD-responsive promoter, as shown in Fig. 4-6C-6D.

Then, we analysed the effect of TAOK1, STK38L and β-TrCP downregulation on CM proliferation. Of notice, siRNAs against both TAOK1 and β-TrCP significantly enhanced EdU incorporation and this effect was abolished when these siRNAs were combined with the specific silencing of YAP; in contrast, the downregulation of STK38l had no impact on cell proliferation (Fig. 4-6E-F).

Taken together, these results demonstrate that the mechanism exploited by miR-199a-3p to induce CM proliferation involves the direct targeting of two proteins, TAOK1 and β-TrCP, which regulate YAP activation via different mechanisms. Further analysis, such as rescue experiments in which CM proliferation is evaluated after miR-199a-3p treatment in presence of TAOK1 and β-TrCP overexpression, will clarify the relevance of these miR-199a-3p targets among the others in terms of CM proliferation.
**FIGURE 4-6. MECHANISM FOR YAP ACTIVATION BY miR-199a-3p.**

**A-B.** Representative western blots of β-TrCP and TAOK1 after CM treatment with the respective siRNAs. Data are mean ±SEM (n=3 independent experiments); *P<0.05, **P<0.01; t-test.  

**C-D.** Downregulation of TAOK1, β-TrCP and STK38L stimulate TEAD transcriptional activity. CMs were transfected with siRNAs against TAOK1, β-TrCP or STK38L in combination with a TEAD luciferase reporter. The results were normalized to those obtained by a constitutively expressed Renilla luciferase plasmid. Data were normalized over a non-targeting (NT) siRNA. Data are mean ±SEM (n=5 independent experiments); *P<0.05; one-way ANOVA.  

**E.** Representative images of CMs stained for sarcomeric α-actinin, EdU incorporation and Hoechst (nuclei) after treatment with siRNAs against TAOk1 and β–TrCP transcripts with a siRNA against YAP or a siRNA NT. Scale bar: 100 µm.  

**F.** Percentage of sarcomeric α-actinin-positive cells that had incorporated EdU were analyzed 72 hr after treatment with specific siRNAs in combination with an anti-YAP or a non-targeting (NT) siRNA. Data are mean±SEM (n=4 independent experiments); *P<0.05, **P<0.01; one-way ANOVA.
4.4. Modulation of the actin cytoskeleton by Cofilin-2 regulates cardiomyocyte proliferation

Transfection of neonatal CMs with the selected microRNAs often resulted in relevant cellular morphological changes, including rounding of the cell surface, as shown in Fig. 4-2C. Consistent with this observation, our RNAseq study revealed that many differentially expressed genes were involved in actin cytoskeleton remodeling (a list of 79 genes known to be involved in the actin cytoskeleton remodeling is shown in Table 2, along with the fold changes upon each miRNAs transfection). Fig. 6-4B shows the genes differentially regulated upon miRNA treatment. Several of the downregulated genes encoded for proteins able to directly bind G-actin and prevent actin polymerization (Xue and Robinson, 2013). In particular, Cofilin2 was downregulated by 5 out of 6 investigated miRNAs; Twinfilin1 (Twf1) by 4 miRNAs and Twinfilin2 (Twf2) by all 6 miRNAs; Thymosin β4 (Tmsb4x) by one miRNA and Profilin2 (Pfn2) by 3 miRNAs. Moreover, 5 out of 6 miRNAs also downregulated Csrp3, a LIM-only domain family protein previously described to be able to bind Cofilin2, enhancing its activity in F-actin depolymerization (Papalouka et al., 2009). Another downregulated target of 4 out of 6 miRNAs is Mical3, a member of the Mical family of proteins also involved in actin depolymerization (Fremont et al., 2017). Finally, Aurora A kinase (Aurka) a serine/threonine kinase playing a critical role during early mitotic stages and spindle formation, known to regulate actin cytoskeleton remodeling through Cofilin phosphorylation (Ritchey and Chakrabarti, 2014), was commonly upregulated by all the investigated miRNAs. Fig. 4-7A shows the actin-related proteins that were also predicted as miRNA direct targets according to the TargetScan software.

In particular, we noticed that the Cofilin2 transcript was downregulated by all the miRNAs under investigation, with the exception of miR-590-3p. Furthermore, Cofilin2 was the predicted target of 4 of these miRNAs (miR-199a-3p, miR-1825, miR-302d and miR-373; Fig. 4-7A). We demonstrated that all these 4 miRNAs were able to directly bind the Cofilin2 3’UTR in UTR-luciferase assays (Fig. 4-7B). Accordingly, from the quantification of Cofilin2 transcript levels in neonatal rat CMs upon miRNA-treatment, a significant downregulation of Cofilin2 transcript was experimentally detected in CMs treated with these 4 miRNAs, in addition to CMs also treated with miR-33b* (Fig. 4-7C). Downregulation of Cofilin2 by miR-33b* might be due to an indirect effect, or an effect mediated by the miRNA binding to Cofilin2 transcript outside the 3’UTR. Analysis of Cofilin 2 protein levels in miRNA-treated CMs provided consistent results showing downregulation of this factor after treatment with miR-199a-3p, miR-1825, miR-302d and miR-373 (Fig. 4-7D).

Collectively, these results indicate that proteins that regulate the actin cytoskeleton dynamics and, in particular, the inhibitor of F-actin polymerization by Cofilin2, are frequently targeted, either directly or indirectly, by miRNAs inducing CM proliferation.
**Figure 4-7. Cofilin2 is a common target of pro-proliferative miRNAs.**

A. Schematic overview of proteins involved in actin cytoskeleton remodeling which are predicted targets of the pro-proliferative miRNAs. B. Results of 3'UTR-luciferase assay to evaluate direct targeting of Cofilin2 (Cfl2) by the miRNAs under investigation. Experiments were performed as in Fig. 4-5C-E. Data are mean ±SEM (n=3 independent experiments); *P<0.05, **P<0.01; one-way ANOVA. C. Real-time RT-PCR quantification of the Cofilin2 expression in cardiac cells after miRNA-treatment. A siRNA against Cofilin2 (siCfl2) was used as a positive control. Data are mean±SEM (n=3 independent experiments); *P<0.05, **P<0.01; one-way ANOVA. D. Effect of miRNAs on Cofilin2 protein levels in neonatal rat CMs. Representative western blot shows downregulation of Cfl2 upon pro-proliferative miRNAs. In the lower panel: quantification of Cfl2 levels. Data are mean±SEM (n=3 independent experiments); *P<0.05, **P<0.01; one-way ANOVA. The red line indicates Cofilin2 levels in CMs treated with the control miRNA, cel-miR-67.
4.5. Remodeling of the actin cytoskeleton in proliferating cardiac myocytes

The actin-severing protein, Cofilin2, regulates actin cytoskeleton dynamics by inducing polymerized actin (F-actin) conversion back into its monomeric form (G-actin). We thus evaluated the ratio between G- and F-actin in cardiomyocytes treated with selected pro-proliferative miRNAs or with a siRNA against Cofilin2; in the presence of this siRNA we obtained a silencing of its target protein of >60% in CMs, which correlated with an upregulation of the levels of F-actin, as shown in Fig. 4-8A. The G/F ratio decreased in all the considered miRNA-treated CMs. Importantly, this ratio reached statistical significance for miR-199a-3p, miR-373, miR-302d and miR-33b* when compared to the negative control. The G/F ratio was also decreased in presence of the Cofilin2 silencing, as shown in Fig. 4-8B and Fig. 4-8C for representative blots and quantification respectively.

Next, we wanted to visualize actin cytoskeleton remodeling in cells treated with some of the pro-proliferative miRNAs or with the anti-Cofilin2 siRNA. For this purpose, we performed immunofluorescence staining using a fluorescein-tagged phalloidin to display F-actin (green). In Fig. 4-8E, in control CMs treated with the C. elegans miR-67, actin organization was detected in long threads throughout the cytoplasm, overlapping with those of the cardiac specific, α-actinin (red). In contrast, a round-shaped morphology i, with actin fibers assembled in gross, round bundles at the cytosol periphery, characterized cells treated with miR-199a-3p and miR-373; these bundles of cortical actin were marked also in α-actinin-negative cells (fibroblasts). CMs treated with the two miRNAs also showed a decreased level of sarcomere organization. On the contrary, no evident morphological effect was detected in cells treated with miR-590-3p. Of notice, the same pattern of F-actin distribution also characterized cells treated with the siRNA against Cofilin2, as shown in Fig. 4-8D. Round-shaped CMs after miRNA and siRNA treatments were evaluated using the cell counter tool of the ImageJ software and the relative quantification are presented in Fig. 4-8F.

Together, these data are consistent with the conclusion that the pro-proliferative miR-199a-3p and miR-373 determine actin cytoskeleton remodeling and, consequently, CM morphology changes through the direct downregulation of Cofilin2.
**FIGURE 4-8. miRNA TREATMENT OF CARDIOMYOCYTES INDUCES REMODELLING OF THE ACTIN CYTOSKELETON**

A. Representative blots indicating that the downregulation of Cofilin2 changes the G/F actin ratio in favour of the actin polymerized form (F-actin). siRNA NT: non-targeting siRNA control. B. Quantification of the G-actin/F-actin ratio in CMs upon pro-proliferative miRNAs or anti-Cofilin2 siRNA treatment. Representative western blots, using an anti-a-actin antibody, of supernatants (containing G-actin) and pellets (F-actin) obtained by ultracentrifugation of lysates from the treated CMs. The G/F ratio is shown at the bottom of each band pair. The positive (+) control was provided by the kit manufacturer's. C. Quantification of the G/F actin ratio (percentage) obtained as in panel B. Data are mean ±SEM (n=3 independent experiments); *P<0.05, **P<0.01; one-way ANOVA. D. Percentage of CMs with a rounded shape (as in the representative images in Panels E and F after treatment of CMs with miR-199a-3p or Cofilin 2 siRNA). Data are from the analysis of over 400 (n=4 independent experiments); shown are mean ±SEM; **P<0.01; one-way ANOVA. E. Immunofluorescence images showing remodelling of the actin cytoskeleton upon miRNA treatment. Fluorescent phalloidin staining (green) marks F-actin, CMs are visualized with an anti-a-actinin antibody (red), nuclei with Hoechst (blue). F indicates fibroblasts (α-actinin negative cells) F. Same as in panel E in CMs treated with an anti-Cofilin2 siRNA or with a nontargeting (NT) siRNA control. Scale bar: 65 μm; Scale bar: 33μm

To evaluate cell cycle progression in cells treated with miR-199a-3p, miR-590-3p, miR-373 or the anti-Cofilin2 siRNA, we performed a phospho-histone H3 staining, as shown in the representative pictures in Fig. 4-9A for the miRNAs and Fig. 4-9B for the siRNA. As shown in Fig. 4-9C, all miRNA treatments induced 5-9% of the cells to move through the G2/M phase. Importantly, mitotic cardiomyocytes showed disruption of the sarcomeric architecture, as concluded from the diffused staining with α-actinin shown in Fig. 4-9D. Finally, different mitotic stages are shown in Fig. 4-9E; CMs are stained with α-actinin. Together, these data show significant morphological changes in CM architecture during cell cycle progression, which are recapitulated by the absence of Cofilin2.
FIGURE 4-9. SARCOMERIC ARCHITECTURE WAS DISRUPTED IN PROLIFERATING CARDIOMYOCYTES UPON BOTH MiR-199A-3P AND COFILIN2 siRNA TREATMENT

A-B. Representative images showing cells in G2/M phase treated with miR-199a-3p (A) or siRNA against Cofilin2 (B), showing nuclear staining of phospho-histone H3 (phospho-H3 (S10), red) and diffused cytosolic staining for α-actin (green). Nuclei are visualized with Hoechst 33342 (blue). C. Percentage of pH3 (S10)+, α-actinin+ cells (CMs) after treatment with the selected miRNAs or anti-Cofilin2 siRNA. Cel-miR-67 and a non-targeting (NT) siRNA served as negative controls for miRNA and siRNA respectively. Data results from the evaluation of over 400 CMs from four different experiments; shown are mean±SEM; **P<0.01, *P<0.01 one-way ANOVA. D. Representative images of G2/M cardiomyocytes (defined as in C), showing nuclear positivity for phospho-histone H3 (pH3(S10), green) and disruption of the sarcomeric architecture, as suggested by the diffused α-actinin staining (red). Nuclei are counterstained with Hoechst 33342 (blue). E. Mitotic CMs in subsequent phases of mitosis (from a to d). Staining corresponds to the one in D.
4.6. Downregulation of Cofilin2 activates cardiomyocyte proliferation through YAP activation

We wondered whether cardiac cell proliferation mediated by Cofilin2 inhibition and actin cytoskeleton rearrangements might require YAP activation. In accordance with this possibility, we found that, in CMs, silencing of Cofilin2 mediated by RNAi determined activation of the TEAD-luciferase reporter as shown in Fig. 4-10A. In line, cardiomyocyte treatment with anti-Cofilin2 siRNA resulted in a marked upregulation of the transcript levels of two well-established YAP-responsive genes, CTGF and CyR61 (Zhao et al., 2008) as shown in Fig. 4-10B. Moreover, co-transfection of CMs with a siRNA against YAP abolished the pro-proliferative effect exerted by Cofilin2 silencing, similar to the inhibitory effect observed in the presence of miR-199a-3p (Fig. 4-10C; representative IF images showing reduction EdU incorporation rate in Fig. 4-10D). The obtained data are indicative of a correlation between the actin polymerization process, which is increased by Cofilin2 inhibition, and YAP activation. To investigate this link, we suppressed actin polymerization using cytochalasin D and analysed the levels of both cytosolic and nuclear YAP1. CMs treated with this drug reduced the levels of nuclear YAP1 in a time-dependent manner, whereas increased the amounts of the cytoplasmic, phosphorylated YAP1 (representative western blots in Fig. 4-10E and quantification in Fig. 4-10F). This effect resulted in the drastic abolishment of the levels of the CTGF and CyR61 gene mRNAs (Fig. 4-10G).

Together, these results demonstrate that, in CMs, the extent of actin polymerization regulates YAP nuclear activity and, as a consequence, controls the levels of proliferating cells.
**Figure 4-10. Perturbation of the actin cytoskeleton activates YAP nuclear translocation and activity**

**A.** Downregulation of Cofilin2 activates TEAD reporter activity. The graph reports the results of TEAD-firefly luciferase reporter analysis of CMs transfected with miR-199a-3p and Cofilin2 siRNA. Experiments were performed as in Fig. 1C. Transfection efficiency was standardized over a Renilla luciferase reporter. Data are mean±SEM (n=4 independent experiments); *P<0.05, **P<0.01; one-way ANOVA. **B.** Real-time RT-PCR analysis of the levels of the two TEAD responsive genes CTGF and Cyr61 in CMs transfected with anti-Cofilin2 siRNA. Data are mean±SEM (n=4 independent experiments); **P<0.01; t-test. **C.** Downregulation of Cofilin2 activates CM replication in a YAP-dependent manner. The graph shows the percentage of α-actinin-positive cells that have incorporated EdU after 72 h-treatment with anti-Cofilin2 siRNA or miR-199a-3p mimic alone or in combination with an anti-YAP siRNA. **D.** Representative pictures showing CMs incorporating EdU after treatment with an anti-Cofilin2 siRNA as in Panel B in the absence or presence of an anti-YAP siRNA. Scale bar: 100µm. **E.** Treatment of CMs with cytochalasin D blocks nuclear translocation of YAP. Representative blots showing the levels of nuclear and cytoplasmic YAP1 and phospho-YAP1 (P-YAP1) in CMs treated with DMSO or cytochalasin D. **F.** Downregulation of Cofilin2 activates nuclear YAP1 independent of phosphorylation. The graph shows the nuclear-to-cytoplasmic ratio of YAP1 and phospho-YAP1 (P-YAP1) in CMs treated with DMSO or cytochalasin D. **G.** Downregulation of Cofilin2 activates nuclear CTGF and Cyr61. The graph shows the nuclear-to-cytoplasmic ratio of CTGF and Cyr61 in CMs treated with DMSO or cytochalasin D.
with cytochalasin D for the indicated time points. GAPDH and p84 were used for loading controls of cytoplasmic and nuclear fractions respectively. F. Quantification of YAP nuclear translocation in CMs treated with cytochalasin D. Data are mean±SEM (n=3 independent experiments); **p<0.01; one-way ANOVA. G. Treatment with cytochalasin D blocks transcription of YAP-responsive genes. The graph shows the levels of the CTFG and CyR61 mRNAs, measured by real-time RT-PCR, in CMs treated with cytochalasin D for the indicated time points. Data are mean±SEM (n=3 independent experiments).

4.7. High throughput screening of FDA-registered drugs identifies the neuroactive alkaloid harmine as a powerful inducer of cardiomyocyte proliferation

In addition to miRNAs, we wondered whether some existing small chemical compounds might also be able to boost CM proliferation, alone or in combination with the pro-proliferative miRNAs miR-199a-3p and miR590-3p. For this aim, we selected a library of 780 FDA-registered compounds (Enzo Life Sciences), which was assembled by the vendor to maximize chemical diversity and thus cover the current full spectrum of pharmacologically active molecules (Table 3).

We performed a high-throughput, fluorescence-microscopy-based, screening in neonatal rat CMs to test these molecules for their ability to induce CM proliferation (Fig. 4-11A). Primary rat neonatal cardiac cells were prepared with a purity about 90% and reverse transfected with miR-199a-3p and miR-590-3p (or cel-miR-67 as a negative control). After 24 h of transfection, CMs were treated with the library of FDA-registered drugs. Each compound was administered at the final concentration of 10 µM (DMSO was used as a control). After 48 h from plating, cells were fixed and stained using the sarcomeric α-actinin to visualize CMs (green), Hoechst for nuclei (blue) and EdU incorporation (red nuclei) was evaluated as read-out of proliferation. The screening was performed in duplicate.

The most effective molecule in boosting cardiac proliferation was the alkaloid harmine, a component of several psychoactive plant extracts (Fig 4-11B, -11C and -11D). Of notice, harmine treatment was able to promote CM proliferation by itself, in the absence of miRNA co-treatment, reaching even higher proliferation levels than miR-199a-3p and miR-590-3p. Simultaneous treatment of cells with harmine and miR-199a-3p did not have synergistic effects; on the contrary, harmine and miR-590-3p treatment determined over 50% of CMs to become EdU-positive (Fig. 4-11E). This is in line with the notion, ensuing from our previous experiments, that miR-199a-3p and miR-590-3p act through largely different pathways.

Representative images of CMs after harmine treatment are shown in Fig 4-11G, -11H and -11I demonstrating a significant increase of the total number of CMs as well as EdU incorporation in CM nuclei. The screening results (Fig.11B-11D) are presented using the Z-score, which shows the distance between the raw score and the population mean in units of standard deviation. When the raw score is below the mean, Z is negative, while it becomes
positive when the raw score is above the mean.

**Figure 4-11. High throughput screening of small molecules identifies the neuroactive alkaloid harmine as a powerful inducer of CM proliferation**

A. Schematic overview of the experimental procedure. A library of 780 FDA-registered compounds was screened in neonatal rat CMs for their ability to induce CM proliferation. Primary cardiac cells were reverse transfected with miR-199a-3p and miR-590-3p (or cel-miR-67 as a control). Twenty-four hour after transfection, CMs were treated with the library of FDA-registered drugs. B-D. Results of the screening are presented in the plot as Z-scores (x axis), in which the Z score represents the distance between the raw score and the population mean in units of the standard deviation. Z is negative when the raw score is below the mean, positive when above. Each dot represents a different drug. E. EdU quantification (%) of in neonatal rat CMs are reported in the graph. Data are mean ±SEM (n=3 independent experiments); *P<0.05; one-way ANOVA F. The Total number of CMs was increased after harmine treatment as shown...
4.8. Harmine treatment induces CM proliferation *in vivo*

Next, we investigated the pro-proliferative effect of harmine on cardiac myocytes *in vivo*. Harmine or physiological solution were administrated via intraperitoneal injections in neonatal CD1 mice (P1) for 12 days at the dosage of 1 mg/kg body weight. After 10 days, a single dose of EdU was injected and, 48 hours later, mice were sacrificed and hearts collected for further analyses (Fig. 4-12A). Immunofluorescence staining was performed on the total hearts, as shown in Fig. 4-12B: EdU incorporation was significant increase in CMs of mice treated with harmine in comparison with the control group (6.7 % EdU+CMs treated mice; 2.5% EdU+CMs controls; **P<0.01; t-test). Of note, the presence of increased septum and left ventricular posterior wall thickness, observed in the harmine-injected mice, was an indication of an increase in cardiac mass. Consistent with effect, there was a trend for an increase in cells positive for the proliferation marker phospho-histone 3 (Fig. 4-12C). Quantification of phospho-H3 positivity is shown in Fig. 12D.

**Figure 4-12. Harmine treatment induces CM proliferation in vivo**

A. Neonatal mice at postnatal day 1 (p1) were injected with harmine or PBS (control) for 5 times, followed by EdU injection at day 10. Hearts were collected after 12 days and stained for EdU. B. EdU and a-actinin staining of murine hearts injected with harmine or PBS. Scale bars, 1mm. C. Phospho-H3 (green), tropomyosin (red) positive cells in...
paraffin sections of hearts treated with harmine or PBS identify proliferating cardiomyocytes. Relative quantification are shown in panel D. Data are mean ±SEM (n=6 mice: 3 controls and 3 treated mice). Hoechst marks nuclei (blue). Bar scale: 250 um

4.9 Harmine promotes heart regeneration in an in vivo model of myocardial infarction

Next, we wanted to assess the regenerative potential of harmine in an in vivo model of myocardial infarction (MI). For this purpose, PBS or harmine (10 mg/kg body weight) were injected in CD1 mice, which were then monitored for a two-month period (Fig. 4-13A). Importantly, since harmine is a neuroactive compound, the drug was administered starting from one week before the permanent ligation of left anterior descending coronary artery, in order for the animals to get exposed to it in advance of treatment.

Two months after MI, harmine-treated mice showed a marked improvement in cardiac contractile function compared to the control group, as shown by a higher left ventricular ejection fraction (LVEF) measured by echocardiography (Fig. 4-13C). Consistent with this finding, infarct size in the harmine-treated mice was smaller (1.8 fold reduction in comparison with the control mice, according to ImageJ software measurements), as shown in representative Sirius Red-stained cardiac cross-sections (Fig. 4-13B). Furthermore, the left ventricles of these mice showed a trend toward reduced levels of dilatation as concluded from the echocardiographic analysis of left ventricle volume at the end of systole and diastole (Figs. 4-13D, -13E).

**Figure 4-13.** The alkaloid harmine promotes heart regeneration in an in vivo model of myocardial infarction

A. Schematic overview of the in vivo experiment. PBS or harmine (10 mg/kg body weight) were injected in CD1 mice, starting from one week before myocardial infarction (MI) and monitored for a period of two months. B. Representative
PicusSirius Red-stained cross-sections of hearts at 8 weeks after MI in control and harmine-treated hearts are shown, to evaluate infarct size and fibrotic scar compared to the control hearts. The arrowheads indicate the infarcted area. C. From echocardiographic data, % LVEF (left ventricular ejection fraction) was evaluated at 1, 2, 4 and 8 weeks after surgery. D-E. Left ventricle dilatation was evaluated by LVESV and LVEDV (left ventricle volume at the end of systole/ diastole) in uL at 0, 1, 2, 4 and 8 weeks.

4.10 Yap-dependent harmine proliferation in neonatal rat CMs

Since from the FDA-registered drug screening it emerged that miR-199a-3p and harmine treatment do not show additive effect on CM proliferation, we reasoned that these molecules might share the same molecular mechanism to promote CM cell cycling. We therefore wondered whether YAP activation was an essential element for harmine to induce CM proliferation. To test this hypothesis, we transfected neonatal rat CMs with the siRNA against YAP1 which was already described above to knockdown the YAP1 transcript. Twenty-four hours before fixing the cells, we treated CMs with harmine and, 4 hours later, with EdU. Analysis of EdU incorporation revealed that the absence of YAP1 in harmine-treated CMs significantly reduced their proliferative ability (Fig. 4-14A and -B), demonstrating the essential role of YAP in CM cytokinesis mediated by harmine. Representative images of proliferating cardiomyocytes upon harmine administration and their inhibition upon YAP downregulation are shown in Fig. 4-14A.

Since harmine is a compound belonging to the alkaloid family, we next wondered whether other alkaloids of the same class, such as harmane and harmaline (Fig. 4-14E), share the same pro-proliferative capacity. We found that the most potent compound inducing cardiomyocyte proliferation was harmine, as shown in Fig. 4-14C and -14D, although a modest increase in cardiac myocyte proliferation was also observed in harmaline-treated cells. The effect of harmaline on CM proliferation can be explained by the similar molecular structure of these two drugs, which both have a methoxy and a methyl group that are not present in harmane (Fig. 4-14E). Finally, we also tested whether these members of the alkaloid family are also able to induce YAP activity, using a TEAD-luciferase reporter assay. The results obtained demonstrated that only harmine promotes YAP activation in CMs (Fig. 4-14F) reaching similar levels as the positive control pYAPS5A (the constitutive activated YAP).
**FIGURE 4-14. YAP-DEPENDENT HARMINE-INDUCED PROLIFERATION IN NEONATAL RAT CMs**

A. Representative images showing proliferating cells transfected with siRNA anti-Yap1 or a non-targeting (NT) siRNA in the presence of harmine treatment. Cells were stained for α-actinin (green) to visualize CMs, EdU staining (red) to identify proliferating cells. Nuclei are visualized with Hoechst 33342 (blue). B. Quantifications of EdU+ CMs (%) are shown in the graph. Data are mean ±SEM (n=3 independent experiments); *P<0.05; t-test. Scale bar: 100µm C-E. Harmine analogues, harmaline and harmane (molecules structure in E), were tested for their ability to induce CM proliferation (C). Quantification of EdU+ CMs (%) are shown in the graph D. Data are mean ±SEM (n=3 independent experiments); **P<0.01; one-way ANOVA. F. CMs were treated with harmine analogues in combination with a TEAD luciferase reporter. Data were analyzed as in Fig. 4-6C. Data are mean ±SEM (n=5 independent experiments); **P<0.01; one-way ANOVA.
4.12 Dyrk1a inhibition and Yap activation mediate the pro-proliferative effect of harmine in cardiomyocytes

It is known that harmine is also able to inhibit the dual specificity tyrosine phosphorylation regulated kinase (Dyrk1a), while both harmane and harmaline specifically inhibit MAO-A (Herraiz, Gonzalez et al. 2010) (Herraiz and Chaparro 2006). We therefore investigated whether the mechanism by which harmine induces CM proliferation might require Dyrk1a kinase inhibition.

To investigate the role of Dyrk1a in the harmine-mediated CM proliferation, we silenced Dyrk1a (>90% protein downregulation; Fig. 4-15C) using a specific siRNA against its mRNA and evaluated EdU incorporation in cardiac myocytes. The results obtained showed that suppression of Dyrk1a was sufficient to induce CM proliferation (Fig. 4-15A), as concluded by the increase in EdU incorporation observed upon Dyrk1a silencing (Figs. 4-15A and -15B). On the contrary, we found that adenovirus-mediated overexpression of Dyrk1a reduced proliferation by approximately 50% (Fig. 4-15D and -15E), correlating with a 50% decreased level of TEAD reporter activity (Fig. 4-15I). The overexpression of Dyrk1a mediated by adenovirus was confirmed by the evaluation of Dyrk1a protein level (2.5 fold changes over control) as shown in the Fig. 4-14F. An adenovirus carrying GFP was used as a control for these experiments. Of notice, TEAD activation levels, boosted by harmine treatment, was markedly reduced when cells were co-treated with harmine and Adeno-Dyrk1a (Fig. 4-15I).

Next, we wanted to understand whether the increased proliferation observed after Dyrk1a silencing was mediated by YAP1. For this aim, we analyzed EdU incorporation in cardiac myocytes after treatment with siRNA Dyrk1a in combination with a siRNA against YAP1. As shown in Figs. 4-15G and 4-15H, YAP1 inhibition reduced the CM proliferation induced by Dyrk1a silencing.

Collectively, these data show that Dyrk1a inhibition is responsible for the boost of proliferation triggered by harmine treatment in CMs.
**Figure 4-15. Dyrk1a inhibition and Yap activation mediate pro-proliferative harmine effects in CMs**

A. Representative images of CM proliferation mediated by siRNA-silencing of Dyrk1a. CMs were transfected with a specific siRNA against Dyrk1a or non-coding siRNA (siRNA nc). CMs are visualized in green as α-actinin-positive cells. Hoechst staining was used for nuclei (blue). EdU incorporation is visualized in red. Scale bar: 100 µm. B. The graph shows the percentage of sarcomeric α-actinin-positive cells that have incorporated EdU. Data are mean ± SEM (n=3 independent experiments); *P<0.01; t-test. C. Western blot showing siRNA-mediated Dyrk1a downregulation. GAPDH was used as loading control. D. Representative images of CMs upon overexpression of Dyrk1a mediated by an adenovirus (AdDyrk1a). An adenovirus carrying GFP, adGFP, was used as a control. CMs are stained same as in A. E. Percentage of sarcomeric α-actinin-positive cells (green) that have incorporated EdU (red nuclei) are reported in the graph. F. Western blot showing overexpression of Dyrk1a protein mediated by the adenovirus. G. Representative images of CMs transfected with siRNA against Dyrk1a or a non-coding siRNA (siRNA nc) in the presence of Yap1 knockdown. H. The graph shows the percentage of sarcomeric α-actinin-positive cells that have incorporated EdU. Data are mean ± SEM (n=3 independent experiments); *P<0.01; one-way ANOVA. I. CMs treated with adDyrk1a alone and in combination with harmine treatment were transfected with a TEAD luciferase reporter. A constitutively expressed Renilla luciferase plasmid was used to normalize the results. Data are mean ± SEM (n=5 independent experiments); *P<0.01; one-way ANOVA.

**4.13 The role of the Dyrk family in promoting CM proliferation**

To understand whether other Dyrk-family members (Fig. 4-16A) had the same capability of Dyrk1a in promoting CM cell cycle activity, we analysed CM proliferation upon transfection with siRNAs against each member of the Dyrk family (Dyrk1a, Dyrk2, Dyrk3, Dyrk4). CMs
were visualized with α-actinin staining (green), EdU staining (red) was used to identify proliferating cells and nuclei were stained with Hoechst 33342 (blue) as shown in Fig. 4-16C. The specific silencing of Dyrk1a was the only treatment able to significantly promote an increase in cardiac myocyte proliferation (Fig. 4-16B).

Next, CMs treated with siRNAs against Dyrk1a, Dyrk2, Dyrk3 and Dyrk4 were transfected with a TEAD luciferase reporter. The results were normalized to those obtained by a constitutively expressed Renilla luciferase plasmid and calculated as fold over control (non-coding siRNA). Even though Dyrk1a alone was able to induce CM proliferation, it was not the only one able to increase Yap-mediated TEAD activity. Indeed, Dyrk2 also seems to have an effect in inducing Yap activation, as reported in Fig. 4-16D.

Finally, the expression of two YAP-responsive genes, CTGF and Birc5, were marked increased upon Dyrk1a silencing or harmine treatment, as shown in Fig. 4-16E and -16F.

**FIGURE 4-16. DYRK1 FAMILY AND YAP EFFECT ON CARDIOMYOCYTE PROLIFERATION**

A. Schematic representation of Dyrk family member structure. NSL= nuclear localization sequence; DH: DYRK-homology box; kinase catalytic domain; PEST: proline, glutamic acid, serine, and threonine rich domain; HIS: poly-
4.14 Cardiac specific Dyrk1a KO induces cardiac proliferation in adult mice

To verify whether Dyrk1a silencing would also promote cardiac cell proliferation in vivo, an inducible mouse model of cardiomyocyte-specific Dyrk1a KO was created (MHC-MerCreMer-Dyrk1a f/f) by crossing a Dyrk1a floxed mouse (obtained from Prof. John D. Crispino, Robert H Lurie Medical Research Centre, US) with an MHC-MerCreMer driver mouse. The induction of Dyrk1a KO specifically in cardiomyocytes was obtained by 5 injections of 4-OH-tamoxifen, which was administrated intraperitoneally in two month-old mice (n=6) at the dosage of 40 mg/kg body weight (experimental scheme Fig. 4-17A).

We verified the cardiac specific Dyrk1a ablation in the hearts of MHC-MerCreMer-Dyrk1a f/f mice and in the Dyrk1a f/f control group (n=6) using Real Time-qPCR Fig. 4-17B. Mice were monitored with echocardiography for functional cardiac parameters such as left ventricular ejection fraction (LVEF), left ventricular mass (LVmass) and left ventricular area of the wall in diastole, LVAWd, at different time points (week 0, 2, 4 and 8 weeks) (Fig. 4-17D, -17E and -17F). We referred to week 0 as the time point just after the injections of 4-OH-tamoxifen (week-1) and before the first EdU injection (week1). EdU was injected in mice from week 1 to the end of the follow-up period (2 months). As shown in Fig. 4-17D and -17F, analysis of the left ventricular parameters of cardiomyocyte-specific Dyrk1a KO mice revealed a significant increase in LVAWd already at week 1 after the induction of the KO phenotype, which, in combination with an increase in LVmass, that becomes significant at week 2, was indicative of an increased thickness of the left ventricular wall without any correlation with an eventual increased in heart weight (Fig.4-17C).

In order to assess whether the observed phenotype was due to hyperhrophic remodelling of CMs or to an increase in CM number, wheat germ agglutinin staining (WGA) was used to identify plasma membranes and measure cardiac cell surface (Fig 4-17G and -17H), while EdU incorporation in α-actinin positive cells was used as a marker of proliferating cells, as
shown in Fig. 4-17I. The results obtained indicated that cardiac proliferation was responsible for the thickness of the heart wall (Fig. 4-17F-17D), since CM size in the Dyrk1a KO mice was not different from that of the control group, as quantification in Fig. 4-17H shows. Collectively, these data show that the cardiomyocyte-specific Dyrk1a ablation leads to increased adult CM proliferation in vivo.

**Figure 4-17. Dyrk1a cardiac specific KO promotes cardiomyocyte proliferation in vivo**

A. Schematic overview of the in vivo experiment. Three doses of 4-OH-tamoxifen were injected in MCM-CRE-\(^{\text{f/f}}\) Dyrk1a mice to induce cardiac Dyrk1a knock out. Mice were monitored for a period of two months. B. Dyrk1a expression levels were evaluated in qRT-PCR in WT and Dyrk1a KO murine hearts. GAPDH was used as housekeeping gene. C. The weight of Dyrk1a WT and KO hearts were measured and normalized on tibia length. D-F. Left ventricular anterior wall thickness in diastolic phase was evaluated at 0, 1, 2, 4 and 8 weeks. E. Left ventricle ejection fraction (LVEF) was evaluating at different time points to monitor heart functionality. F. Left ventricle mass, expressed in mg, was evaluated at 0, 1, 2, 4 and 8 weeks. G. Representative images of WGA staining showing CM area in WT and Dyrk1a KO hearts. H. Quantification of the relative cross-sectional area in WT and Dyrk1a KO mice. I. EdU staining (red) of tropomyosin positive cells (green) in paraffin sections of heart tissue of Dyrk1a KO or WT mice. Scale bar: 100µm.
5 DISCUSSION

The scanty information so far available on the molecular mechanisms that regulate cardiomyocyte cell cycle progression and those that are involved in physiological regeneration during the embryonic, fetal and early neonatal life still hamper the development of novel therapeutic approaches for the vast number of patients suffering of post-myocardial infarction heart failure. In the present work, we investigated the mechanism of action of two distinct pro-proliferative stimuli able to promote cardiomyocyte cell cycle re-entry: a series of microRNAs, identified in our previous work for their ability to stimulate neonatal rodent CM proliferation, and the neuroactive compound harmine, which had emerged from a high throughput screening using a library of FDA-registered drugs.

Our results clearly indicate that signaling from all investigated, pro-proliferative miRNAs converges towards nuclear translocation of the YAP transcriptional co-activator. This final molecular outcome is achieved through the modulation of different pathways, among which the regulation of actin cytoskeleton dynamics plays an essential role.

The Hippo-Yap signaling pathway is a master biochemical cascade in the control of organ size, as shown in YAP overexpression experiments (Camargo, Gokhale et al. 2007) or Hippo core component knock-down (Heallen, Morikawa et al. 2013). In particular, multiple evidence over the last few years has shown that this pathway is an essential regulator of physiological cell proliferation during normal heart development (Xiao, Leach et al. 2016). In adult hearts, YAP overexpression induces CM cell cycle re-entry (Lin, von Gise et al. 2014), while in the case of myocardial infarction, YAP activation drives heart tissue regeneration (Heallen, Morikawa et al. 2013) (Xin, Kim et al. 2013).

Our results, showing that the downregulation of YAP expression by RNAi blunts the pro-proliferative effect of all the 10 top effective miRNAs originally identified to stimulate CM proliferation, confirm that this pathway is also crucial for the exogenous stimulation of proliferation of post-natal CMs. This conclusion is reinforced by the observations that these pro-proliferative miRNAs promote the activation of a TEAD-responsive promoter, increase YAP-responsive gene expression and trigger nuclear import of active YAP.

Given the above discussed link between pro-proliferative microRNAs and YAP signaling, we investigated the molecular targets of these miRNAs, the downregulation of which might be eventually responsible for YAP functional activation in cardiomyocytes. Transcriptomic analysis of CMs in response to miRNA transfection revealed that 6 of the investigated miRNAs act through distinct pathways to achieve YAP activation. Indeed, all 6 of them determined downregulation of at least one of the kinases known to inactivate YAP (STK38L, LATS1/2 and TAOK1). Our results
further confirm previous findings from the Morrisey laboratory, showing that the miR-302/367 cluster - in our work, we analyzed miR-302c and miR-302d, belonging to this family - induces activation of the Hippo pathway in cardiac cells through the downregulation of MST1, MOB1b and LATS2 (Tian, Liu et al. 2015) MiR-199a-3p instead directly targets the 3’UTR of TAOK1 and β−TrCP, the latter acting as the E3 ubiquitin ligase driving YAP degradation through the proteasome (Zhao, Li et al. 2010) . Conversely, we found that the 3’UTR of STK38L is not directly bound by MiR-199a-3p: its downregulation upon miR-199a-3p, miR-302d and miR-373 transfection suggests either an indirect effect or an effect mediated by miRNA binding outside the 3’UTR region of the STK38L transcript.

Of interest, individual downregulation of either TAOK1 or β−TrCP by RNAi was sufficient to stimulate CM proliferation, indicating that the effect of miR-199a is likely the combinatorial outcome of downregulation of these (and likely other) genes, possibly impacting on the same pathway. Finally, consistent with their positive effect on the Hippo pathway, all the tested miRNAs upregulated the levels of the transcriptional enhancer factor TEF-4, TEAD2. This transcription factor belongs to the TEAD family: this isoform is highly expressed in muscles and mediates YAP coactivation of genes involved in DNA replication and cell cycle progression (Kapoor, Yao et al. 2014) (Zhao, Caretti et al. 2006).

Another molecular signature shared by most of the investigated miRNAs was the modulation of the actin cytoskeleton network. In particular, the levels of several factors preventing actin polymerization were significantly downregulated upon miRNA transfection in CMs: these included Cofilin2, Twinfilin1/2, Thymosin β4 and Profilin2. These regulatory proteins are important to maintain an active monomeric (G-actin) pool and prevent filamentous (F-actin) nucleation events (Xue and Robinson 2013). Few miRNAs downregulated Csp3, a Cofilin2-binding protein that enhances Cofilin2-dependent F-actin depolymerization (Papalouka, Arvanitis et al. 2009) and Mical3, known to destabilize F-actin, inhibiting local actin assembly (Fremont, Romet-Lemonne et al. 2017). Downregulation of these factors shifts the balance between F- and G-actin towards the polymerized state, as we observed in the case of transfection of individual miRNA mimics in cardiac cells, in particular miR-199a-3p, miR-302d, miR-373 and miR-33b*. Consistent with this effect, cells treated with miR-199a-3p or miR-373 macroscopically showed a rounded shape and displayed formation of layers of cortical, polymerized actin in bundles close to the cytoplasm periphery.

Interestingly, all the miRNAs we investigated, with the exception of miR-590-3p, were able to down-regulate the transcript of Cofilin2. This protein belongs to the muscle-specific ADF/Cofilin family, essential to modulate the equilibrium of actin assembly, thus being involved in all aspects of cell motility, locomotion and invasion (Bernstein and Bamburg 2010) (Bravo-Cordero, Magalhaes et al. 2013). In particular, Cofilin causes depolymerization of actin filaments, preventing their reassembly (Ghosh, Song et al. 2004). We found that four of the investigated
miRNAs (miR-199a-3p, miR-1825, miR-302d and miR-373) directly targeted the 3’UTR of Cofilin2 transcript. Even more notably, silencing of Cofilin2 by a specific siRNA was sufficient to stimulate CM proliferation and drive morphological changes in the treated cells, in terms of cortical actin accumulation and shape, which were superimposable to the phenotype triggered by miRNA transfection. We also found that the downregulation of Cofilin2 triggered YAP nuclear translocation with consequent activation of its transcriptional pathway, while inhibition of actin polymerization by cytochalasin D blocked this effect. Our results are perfectly consistent with previous findings showing that actin cytoskeleton dynamics is a strong activation signal for YAP in response to mechanical cues from the extracellular environment (Dupont, Morsut et al. 2011).

Keeping with the above experimental results and considerations, there is evidence that YAP activity is promoted and maintained by polymerized F-actin, essential for the maintenance of cellular homeostasis, resulting from a combination of extracellular hits affecting cell morphology and shape (Dupont, Morsut et al. 2011). The interplay between YAP activation and the cytoskeletal arrangement of CMs has an additional layer of complexity, since recent work has shown that YAP directly regulates genes encoding for proteins that promote F-actin polymerization and link the actin cytoskeleton to the extracellular environment, including components of the dystrophin glycoprotein complex (Morikawa, Zhang et al. 2015). Recent data also demonstrate that a component of this complex, Dag1, directly binds YAP and inhibits its pro-proliferative function (Morikawa, Heallen et al. 2017). Since the dystrophin glycoprotein complex connects the extracellular matrix to the actin cytoskeleton, it is conceivable that perturbing integrity of this connection at different levels might lead to YAP activation. This possibility is consistent with our observation that the individual Cofilin2 knockdown, which promotes actin rearrangement, is sufficient to activate YAP.

As far as miR-590 is concerned, although Cofilin2 was not a direct target of miR-590-3p, we cannot exclude that this miRNA interferes with cytoskeleton remodelling in exerting its pro-proliferative effect. Indeed, from the TargetScan prediction, in line with our RNaseq data, miR-590-3p downregulates the cysteine and glycine-rich protein 3 (Csrp3) mRNA levels, which encodes for the Muscle LIM Protein (MLP). As previously mentioned, this protein is involved in the maintenance of cytoarchitecture integrity of muscle cells and increases actin depolymerisation in a Cfl2-mediated manner (Papalouka, Arvanitis et al. 2009). We therefore can speculate that, although miR-590-3p is not directly affecting Cfl2 expression, it most likely acts on actin cytoskeleton remodeling through the silencing of Csrp3, in order to alter G-/F-actin balance to allow CM proliferation.

Recently, the study of actin dynamics has also unveiled a role of nuclear actin in the regulation of cellular transcriptional activity. In this context, additional molecular mechanisms of CM cell cycle regulation could be revealed by exploring the role of nuclear actin upon miRNA treatment, as it would shed lights in the understanding whether it is able to directly affect CM transcriptional activity in the proliferative effect exerted by microRNAs.
Finally, the interconnection between cytoskeletal dynamics, CM replication and YAP activation is further corroborated by the observation that all the pro-proliferative microRNAs under examination activated expression of Aurora A kinase, a protein that regulates G2/M phase transition during the cell cycle (Nikonova, Astsaturov et al. 2013). On the one hand, this serine/threonine kinase phosphorylates Cofilin at multiple sites during early mitosis resulting in the inactivation of its actin depolymerizing function (Ritchey and Chakrabarti 2014). On the other hand, recent work in cancer cells indicates that Aurora A kinase also acts as a direct, positive regulator to enhance YAP-mediated transcription (Chang, Yamaguchi et al. 2017).

In this work we also aimed at identifying small molecule drugs able to boost CM proliferation and thus potentially rescue myocardial function after ischemic injury. A library of 780 FDA-registered compounds was chosen for our screening, which was assembled by the vendor (Enzo) in order to ensure maximum heterogeneity in the chemical and pharmacological spectrum among all the available compounds. Our high-throughput, small molecule screening identified the neuroactive aholoidal drug harmine, as the most potent inducer of CM proliferation.

Harmine, is a small molecule historically isolated from *P. harmala* and *B. Caapi* and belonging to beta-carboline alkaloids family. Harmine was traditionally used in hallucinogenic preparations in tribal ceremonies in the Central Asia, Middle East and South America. Besides its hallucinating properties of harmine, an interesting series of pharmacological effects have been attributed to this drug, including as anti-inflammatory, anti-microbial and anti-psychotic activities (reviewed in (Patel, Gadewar et al. 2012).

In the more recent literature, several papers report that harmine functions as a potent anti-tumor compound. In particular, in Hep3b and HuH7 hepatic cancer cell lines, harmine interferes with the double-stranded DNA break repair mechanism of cancer cells, which naturally allow these cells to survive and rapidly divide. In this context, harmine negatively affects the homologous recombination pathway by inhibiting the recruitment of Rad51 and consequentially determines failure of DNA damage repair mechanisms, with cytotoxicity as the ultimate result (Zhang, Zhang et al. 2015). Moreover, harmine was reported to inhibit tumor growth in head and neck squamous cell carcinoma (HNSCC) by inhibiting Dyrk1a-mediated Foxo3 phosphorylation, thereby promoting cell death (Radhakrishnan, Nanjappa et al. 2016).

In light of the above reported, known effect of harmine, it was quite surprising to us to discover that the drug was a powerful inducer of CM proliferation, in combination with miR-199a-3p or miR-590-3p, but also by itself. Moreover, *in vivo* and in neonatal mice harmine induced CM proliferation already at a dosage of 10mg/kg body weight. In addition, we found that harmine treatment significantly improve cardiac function after the induction of a myocardial infarction in mice, showing its beneficial potential in the heart regenerative process.

In considering the literature, however, one should mention that harmine was already reported to also stimulate proliferation of human neural progenitor cells in vitro (Dakic, Maciel et al. 2016) as...
well as that of pancreatic β-cells, both in \textit{in vitro} and \textit{in vivo} (Wang, Alvarez-Perez et al. 2015). In both cases, inhibition of the ubiquitous kinase Dyrk-1a was found to mediate the proliferative effect of the drug. In particular, in pancreatic β-cells, inhibition of Dyrk1a was shown to result in NFAT nuclear translocation, which in turn induced expression of genes that stimulate cell cycle progression, such as c-Myc.

Our experimental data indicate that, among the alkaloid family members, harmine was the only tested compound able to promote CM proliferation, while the harmine analogues harmaline and harmane, which share a very similar biochemical structure with harmine, did not exert any significant effect.

The Dyrk1a gene is located on human chromosome 21, in the Down syndrome critical region (Shindoh, Kudoh et al. 1996) and is highly expressed in different tissues, such as brain and heart. It is considered to be crucial during early development of the nervous system and a master regulator in brain growth (Guedj, Pereira et al. 2012). Although, in neural cells, Dyrk1a has been described to regulate cell cycle progression and apoptosis, the detailed Dyrk1a downstream molecular mechanism still remains a topic of debate (Fernandez-Martinez, Zahonero et al. 2015). As for harmine, function of Dyrk1a seems to be dependent on the specific cell type and the cell proliferating status.

As for harmine, function of Dyrk1a seems to be dependent on the specific cell type and the cell proliferating status.

The role of Dyrk1a in cardiac myocytes is under investigation in various laboratories. One study has described Dyrk1a as a regulator of cardiac hypertrophy (Kuhn, Frank et al. 2009). Dyrk1a inhibition, mediated by a recombinant adenovirus carrying synthetic miRNAs targeting the kinase mRNA, was shown to induce \textit{in vitro} cardiomyocyte hypertrophy induced by a 24 hr-pulse of phenylephrine. In addition, overexpression of Dyrk1a (AdDyrk1a) was reported to completely rescue the hypertrophic phenotype \textit{in vitro}, while an inactive mutant of Dyrk1a (AdK188R) was ineffective. These findings were explained by the correlation between a decrease in Dyrk1a activity and the increased level of nuclear NFAT, the downstream transducer of calcineurin signaling responsible for CM hypertrophy.

There is an apparent contradiction between the above reported findings and those we obtained regarding the role of Dyrk1a as a negative regulator of CM proliferation. Indeed, we clearly observed that RNAi-mediated silencing of Dyrk1a stimulated CM proliferation without exerting obvious effects on either CM size or hypertrophic fetal gene expression. Differences in the experimental conditions and in timing of observation might explain these different results. In our experiments, Dyrk1a silencing was obtained by using a specific siRNA, which requires at least 72 hr to be effective, while the inactive mutant of Dyrk1a used by Hille and colleagues was evaluated 24 hr after transduction, which is a too short timeframe to appreciate any proliferative events in CMs. In addition, a recent line of thought postulates that CM hypertrophy and proliferation are different sides of the same coin, which depends on the status of the cell that receives a given stimulation. In particular, CMs might require an increase in size (hypertrophic phase) before division; if adult CMs receive a pro-proliferative stimulus, they fail to proliferate and become
hypertrophic. Conversely, neonatal CMs are still capable to accomplish cell division therefore, once exposed to the same kind of stimulation, they overcome the “hypertrophic phase” and complete their mitosis.

The finding that harmine activates CM proliferation by targeting Dyrk1a raises the obvious question of which is the molecular mechanism by which this kinase controls cell proliferation. In this respect, the observation that harmine treatment did not exert any addictive effect with that of miR-199a-3p is consistent with the possibility that both the drug and this miRNA act through similar molecular mechanisms. Consistent with this possibility, we indeed found that harmine treatment resulted in YAP activation and that YAP was essential to mediate the pro-proliferative effect of the drug, again highlighting the essential role of the Hippo/YAP pathway in the regulation of cell cycle progression in CMs. However, how does Dyrk1 inhibition end up in YAP activation still remains a very interesting matter for future investigations. One might speculate that the kinase directly phosphorylates YAP thus inhibiting its activity or that, alternatively, it activates other known upstream kinases in the Hippo pathway, which are known to exert a negative role on YAP. Both possibilities appear to be experimentally testable.

The conclusion that Dyrk1a is an inhibitor of CM proliferation is fully consistent with the findings we observed in vivo in the Dyrk1a conditional knock-out mice. Full Dyrk1a knock-out is known to be embryonically lethal, while its overexpression during embryogenesis results in cardiovascular abnormalities (Fotaki, Dierssen et al. 2002). In our experiments, adult animals in which Dyrk1a was inactivated specifically in the heart, using tamoxifen-inducible Cre under the control of the MHC promoter, showed a significant increase in cardiomyocyte proliferation, with consequent hyperplastic increase in cardiac ventricle mass. These results might appear substantially different from those reported by the De Windt group in a haploinsufficient Dyrk1a mouse, in which pathologic hypertrophic growth after TAC was observed (da Costa Martins, Salic et al. 2010). However this discrepancy is only apparent, since the animal models used in the two studies are substantially different. Da Costa Martins and collaborators, to avoid embryonic lethality, took advantage of a haploinsufficient Dyrk1a mouse model, which is substantially different from the inducible cardiac specific knock-out used in our studies. Additionally, in the da Costa Martins study, analysis was performed at one week after transverse aortic constriction, while, in our case, mice were analysed in the absence of damage.

The effect of Dyrk1a overexpression was also recently studied in the heart (Hille, Dierck et al. 2016), showing that cardiac specific Dyrk1a transgenic mice developed dilated cardiomyopathy. In the same study, the hearts of neonatal transgenic mice, overexpressing Dyrk1a, presented decreased levels of CM proliferation, in agreement with our conclusions. At the molecular level, Hille et al. defined a new role for Dyrk1a as a negative regulator of Rb/E2F signalling mediated by cyclin-D and thus of cardiac myocyte proliferation. These conclusions appear to be fully consistent with our observation that Dyrk1a knock-out significantly increases CM proliferation.
Conclusions and translational perspectives

The discovery that individual miRNAs regulate post-natal CM proliferation is of particular interest towards the potential utilization of these molecules to promote cardiac regeneration. The identification of the molecular targets of the pro-proliferative miRNA is obviously important in this context. The results presented in this Thesis indicate that the stimulation of CM proliferation by miRNAs is a process that requires downregulation of multiple targets, with likely additive functions. In this respect, the pleiotropic effect of miRNAs appears to be of significant advantage compared to the use of single genes or siRNA knockdowns, provided, obviously, that the potential deleterious effects are minimized. For example, miR-199a-3p, one of the most effective miRNAs both ex vivo and in vivo, targets directly the 3’UTRs of Cofilin2 (modulation of actin cytoskeleton), TAOK1 (activation of the YAP inhibitory kinases) and β–TrCP (reduction of YAP degradation), in addition to the 3’UTRs of Hopx1 (a suppressor of embryonic CM proliferation (Trivedi, Zhu et al. 2010) and Homer1 (involved in the regulation of calcium signaling in cardiac cells (Jardin, Lopez et al. 2013), as shown in our previous work (Eulalio, Mano et al. 2012); other mRNAs are likely to be targeted as well. Despite these pleiotropic gene downregulation, however, it is of notice that all the proproliferative pathways appear to converge, and require, YAP activation. This conclusion is consistent with our previous observation, unexplained at the time, that combinatorial combinations of the four most effective miRNAs in our original HTS screen did not exert an effect that was superior to that of each individual miRNA when used at the same concentration of the combined molecules (Eulalio, Mano et al. 2012).

The pleiotropic function of miRNAs stimulating cardiac proliferation suggests that these molecules should be considered with caution in translational terms. While the permanent expression of miR-199a and miR-590 pri-miRNAs using AAV9 vectors in the mouse did not result in apparent deleterious effects (Eulalio, Mano et al. 2012), permanent expression of the miR-302/367 cluster led transgenic animals to develop cardiac dysfunction due to CM hyperproliferation coupled with cell de-differentiation (Tian, Liu et al. 2015). This observation is consistent with the finding that miR-302/367 enhances the reprogramming efficiency of mouse embryonic fibroblasts into induced pluripotent stem cells (iPSCs) (Judson, Babiarz et al. 2009) (Li, Yang et al. 2011). The pleiotropic effect of miRNAs and these observations, however, do not rule out that appropriate dose and schedule for the delivery of synthetic miRNA mimics would be effective and well tolerated. Available evidence already shows that single dose, systemic injection of miR302b/c itself (Tian, Liu et al. 2015) or intracardiac injection of miR-199a-3p mimics (Lesizza, Prosdocimo et al. 2017) determine significant beneficial effect in terms of recovery of myocardial tissue and function. These observations warrant further translation of these findings to large animals first and to the clinics later.

As far harmine is concerned, the potential beneficial effects of this molecule in the clinics was considered for different therapeutic approaches (Patel, Gadewar et al. 2012, Moloudizargari, Mikaili et al. 2013). In this regard, it is important to emphasize that pro-proliferative compounds, in
general, have the potential to promote cell cycle progression in a non-specific way, which could stimulate tumor growth at off-target sides. Harmine, however, appears to be quite peculiar in this respect, since its pro-proliferative effects are exerted in tissues with low proliferative capacity, such as brain, β-cells and heart in our study. While this would be reassuring in terms of safety, however, it still needs to be considered that harmine itself exerts neuroexcitatory effects, which would prevent its direct clinical use. Other alkaloid compounds derived from harmine, might instead prove equally effective in the downregulation of Dyrk1a but devoid of effects on the central nervous system and thus be more apt for clinical use. Thus, there is a translational perspective for this molecule, which would obviously require additional careful experimentation.
References


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Table 1. Hippo-related genes. An update catalogue of genes known to have a role in the regulation of YAP-Hippo pathway are reported in the table.
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<td>Runx family member, transcription factor 1</td>
<td>ENSRNOG00000012543</td>
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<tr>
<td>Kif23</td>
<td>Kinesin family member 20B, cell division cycle</td>
<td>ENSRNOG00000019822</td>
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<tr>
<td>Ets2</td>
<td>ETS2 transcription factor 2</td>
<td>ENSRNOG00000001924</td>
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<tr>
<td>Yap1/Wwtr1</td>
<td>coactivators of transcriptional activity</td>
<td>ENSRNOG00000019195</td>
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</table>

**Expression Values (Log2 Fold Change)**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression Values</th>
<th>Log2 Fold Change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gadd45b</td>
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</tr>
<tr>
<td>Mre11a</td>
<td></td>
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<tr>
<td>Runx2</td>
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<tr>
<td>Kif23</td>
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<tr>
<td>Ets2</td>
<td></td>
<td></td>
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<tr>
<td>Yap1/Wwtr1</td>
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</tbody>
</table>

**Transcriptional Targets**

- Yap1/Wwtr1 are coactivators of transcriptional activity

**PMIDs**

- [26258633]
- [17110958]

**Table:**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression Values</th>
<th>Log2 Fold Change</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
<td>Gadd45b</td>
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<td>Runx2</td>
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<tr>
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</table>

**PMIDs**

- [26258633]
- [17110958]
## Table 2. Actin-related genes. An update catalog of genes known to be involved in the actin cytoskeleton remodeling is shown in the table below. The fold changes (log2) of gene expression (RPKM) upon transfection of miR-1825, miR-199a, miR-302d, miR-373, miR-590 are reported here along with the bioinformatic predictions (TargetsScan software); cel-miR-67 was used as control.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Function</th>
<th>Log2 Fold Change</th>
<th>Bioinformatic Predictions</th>
<th>Literature References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tmsb10</td>
<td>regulator and myosin capping actin protein</td>
<td>-0.17</td>
<td>lower</td>
<td>816439, 163422, 157604, 179751, 85702, 111115, 97523, 297308, 297307, 297309</td>
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<tr>
<td>Arpc1a</td>
<td>actin-related protein</td>
<td>-0.18</td>
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<tr>
<td>Tmod1</td>
<td>tropomodulin 1</td>
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<tr>
<td>Dstnl1</td>
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<td>Wasf1</td>
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<tr>
<td>Fmn2</td>
<td>WASp protein family, actin component in the cholesterol metabolic pathway</td>
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<tr>
<td>Was</td>
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</tr>
</tbody>
</table>

**Footnotes:**
- **Gene Symbol:** Gene symbol used for each gene.
- **Function:** Function of the gene in actin cytoskeleton remodeling.
- **Log2 Fold Change:** Log2 fold change of gene expression upon transfection.
- **Bioinformatic Predictions:** Predictions from bioinformatic tools.
- **Literature References:** References to publications where the gene's role in actin cytoskeleton remodeling is discussed.
<table>
<thead>
<tr>
<th>gene</th>
<th>ENSRNOG00000026212</th>
<th>ENSRNOG00000015496</th>
<th>ENSRNOG00000016731</th>
<th>ENSRNOG00000032443</th>
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<tbody>
<tr>
<td>gene name</td>
<td>tropomyosin 1, alpha</td>
<td>WAS protein family nucleation factor 2</td>
<td>nucleation factor 1</td>
<td>monooxygenase, (translocation) 1</td>
<td>spire-type actin</td>
<td>tropomyosin 4</td>
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<tr>
<td>literature</td>
<td>PMID: 25090971</td>
<td>PMID: 19752190</td>
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<td>creates new nucleation cores with their G-</td>
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<td>tandem-monomer-binding</td>
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<tr>
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</table>

Table 3. FDA-registered drug screening results. Each drug is characterized by the FDA-registered drug name and the compound with which it is associated. The z-score mean value obtained by the evaluation of EU-DMs in changes over control (for column 1=miR-67, column 2=miR-199a-3p, column 3=miR-30a-5p) and column 4=miR-30b-5p) are shown.

### Table 3: FDA-registered drug screening results.

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<tr>
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</tr>
<tr>
<td>hsa-miR-30a-5p</td>
</tr>
<tr>
<td>hsa-miR-590-5p</td>
</tr>
</tbody>
</table>