In this thesis we addressed the involvement of diminished cardiac thyroid hormone (TH) action in the development of chronic heart failure, with special emphasis on the role of the TH-degrading enzyme deiodinase type III (DIO3). In this introduction we will briefly summarize the causes and consequences of pathological cardiac remodeling as well as our current understanding of TH metabolism and action in the heart, which gave rise to the specific aims of this study.
Part I
Cardiac Remodeling and Chronic Heart Failure

Chronic heart failure is in many cases the final stage of cardiovascular disease (CVD) and is characterized by the inability of the heart to provide adequate blood flow to meet the metabolic requirements of tissues. This leads to severe fatigue, breathlessness and may ultimately result in death due to multi-organ failure or arrhythmias. Although various therapeutic interventions may alleviate the symptoms, as yet there is no effective cure. The prevalence of heart failure worldwide continues to increase as a result of increasing prevalence of CVD risk factors, such as: obesity, diabetes, hypertension, and physical inactivity. Moreover, because heart failure becomes more common with increasing age, the number of affected individuals is rising with the ageing global population. Currently, an estimated 23 million people worldwide are affected by chronic heart failure, presenting a major burden on the healthcare system and society (Bui, Horwich, and Fonarow 2011). The single most important cause of CVD-related death is ischemic heart disease. Worldwide over 7 million people die of ischemic heart disease each year, accounting for 25% of CVD-related deaths. This number will increase to an estimated 9 million in 2020 (Mackay and Mensah 2004). This makes ischemic heart disease, which includes myocardial infarction (MI), the current and future leading cause of morbidity and mortality. In the Netherlands, mortality data show that CVD accounted for approximately one out of every four deaths. In 2012, 6195 people died as a result of acute MI, i.e., occlusion of a coronary artery causing ischemia of the downstream myocardium. Another 3525 people succumbed to chronic heart failure due to ischemic heart disease (Leening et al. 2014). The latter group includes a growing number of patients who survived the MI thanks to improved emergency care. It is the process of initially adaptive remodeling following the MI, but subsequent development of chronic heart failure, that is the focus of our research.

A consensus statement defined cardiac remodeling after ischemic injury as the molecular, cellular, and interstitial changes affecting size, shape, and function of the heart (Cohn, Ferrari, and Sharpe 2000). This process of cardiac remodeling is aimed at normalizing the increase in wall stress, due to either pressure and/or volume overload, and maintaining proper cardiac output. It takes a plethora of mechanisms initiated in the infarcted and non-infarcted, i.e., spared myocardium to achieve this (reviewed by (Sutton and Sharpe 2000). Ventricular remodeling is part of a set of compensatory responses following the loss of a critical number of functional cardiomyocytes. These include: enhancement of contractile force (the Frank-Starling mechanism), activation of the sympathetic nervous system (SNS) and renin-angiotensin-aldosterone system (RAAS), increased natriuretic peptide release, and finally cardiomyocyte hypertrophy (reviewed by Drexler and Hasenfuss 2010) [Fig. 1]. However, these initially adaptive responses are not beneficial in the long-term, resulting in chronic heart failure.

I.I Myocyte loss and Frank-Starling mechanism

Immediately following the loss of contractile function in the infarcted area, left ventricle (LV) preload increases. The concomitant additional stretching of the viable myocardium results in an increase in contractile force via the Frank-Starling mechanism, which in the short-term improves cardiac output (Chatterjee and Fifer 2011). Post-infarct remodelling starts with the necrosis of affected cardiomyocytes, scar formation and thinning and expansion of the infarcted area. Recruited inflammatory cells release cytokines and proteolytic enzymes, such as matrix metalloproteases, that cause collagen degradation (Janicki et al. 2004). As a consequence the structural support is lost, which allows cardiomyocytes to rearrange (Whittaker, Boughner, and Kloner 1991; Weisman et al. 1988). This so called cellular ‘slippage’ results in wall thinning and further dilation of the non-infarcted myocardium.
I.II Neurohormonal response – Sympathetic activation

Whereas the dilated ventricle enhances its contraction secondary to elevated preload and the Frank-Starling mechanism to preserve cardiac function, SNS activation is a response to the decrease in cardiac output. Hypotension, which is sensed by baroreceptors in the carotid sinus and aortic arch, triggers sympathetic outflow to the heart (norepinephrine, NE). Through stimulation of β-adrenergic receptors in the spared myocardium, cardiomyocyte force and frequency of contraction is increased, with a concomitant increase in energy turnover. Together with the peripheral vasoconstriction as a result of increasing systemic NE levels, blood pressure and perfusion of vital organs is maintained. However, chronically increased cardiac sympathetic drive cannot be maintained by the heart and in the long run may result in contractile dysfunction and cardiac arrhythmias (Drexler and Hasenfuss 2010; Chatterjee and Fifer 2011).

I.III Activation of the renin-angiotensin-aldosterone system

As a consequence of decreased renal perfusion secondary to the low cardiac output and the direct activation of the renal sympathetic adrenergic system, the RAAS system is activated. Renin is released by the kidney and is responsible for the conversion of angiotensinogen to angiotensin I (ANGI), which is subsequently converted to the vaso-active peptide angiotensin II (ANGII) by angiotensin converting enzyme (ACE). ANGII action is mediated by binding to the specific ANGII receptor, AT1. It includes systemic vasoconstriction and stimulation of the release of aldosterone from the adrenal cortex. Aldosterone is responsible for the increase in sodium and fluid retention, resulting in increased blood volume. Together, RAAS activation and the resulting increased vasoconstriction and blood volume increases afterload, which again increases energy expenditure. In overt heart failure, sustained RAAS activation contributes to the onset of pulmonary edema (Drexler and Hasenfuss 2010).
I.IV Natriuretic peptides  The vasoconstrictive actions are to some extent counterbalanced by the release of atrial natriuretic factor (ANF, mainly from the atria) and brain natriuretic peptide (BNP, from the ventricles) in response to the increase in wall stress. The effect of these peptides is a combination of peripheral vasodilation and natriuresis. However, as HF progresses, the effects of ANF and BNP are attenuated, tipping the balance towards vasoconstriction (Drexler and Hasenfuss 2010).

Together, the increased force production and increased heart rate as a result of sympathetic stimulation of the non-infarcted myocardium, and the additional changes in filling of the ventricles result in improved cardiac pump function. However, over time, these initial adaptive responses are associated with progressive cardiac dysfunction and heart failure (Sutton and Sharpe 2000).

I.V Fibrosis  The loss of cardiomyocytes due to necrosis or apoptosis following ischemia results in replacement fibrosis and further LV dilation. In advanced heart failure, endothelial dysfunction contributes to the onset of a complex mechanism responsible for interstitial fibrosis, which results in increased myocardial stiffness. This is associated with the development of a diastolic component of dysfunction, adding to the already existing impairment of systolic function (reviewed by Paulus and Tschöpe 2013). Together with a small contribution of concentric hypertrophy [see also section I.VI] of viable myocardium, increased collagen deposition also results in reduced capillary density and increased oxygen diffusion distance (Drexler and Hasenfuss 2010). Indeed, inhibiting collagen deposition in transgenic mice improved post-MI cardiac function (van Rooij et al. 2008).

I.VI Cardiomyocyte hypertrophy  The later phase of post-MI remodeling is characterized by, primarily, eccentric hypertrophy of the non-infarcted tissue as a consequence of the persisting volume overload (Drexler and Hasenfuss 2010). Cardiomyocyte hypertrophy is the result of wide range of triggers initiating numerous signal transduction pathways that play a part in cellular growth and gene expression regulation [see also section I.VIII] (Sutton and Sharpe 2000; Bernardo et al. 2010; Kehat and Molkentin 2010; Frey and Olson 2003). This involves the activation of transcription processes, chromatin remodeling and post-transcriptional regulation (Dirkx, da Costa Martins, and De Windt 2013). Hypertrophy is the ultimate response aimed at compensating MI-induced cardiac stress. Although compensation may initially be successful, hypertrophy in the context of post-MI remodeling is considered to be the single most important risk factor for disease progression and mortality (Rame and Dries 2007). Specific changes in gene expression are thought to underlie the ultimate cardiac dysfunction and a notable characteristic of this pathological hypertrophy is the partial recapitulation of the fetal gene program.

Hypertrophy has classically been subdivided as concentric or eccentric. Concentric hypertrophy, following persistent pressure overload, is initiated through pathological stimuli such as hypertension and aortic stenosis, produces an increase in systolic wall stress. This form of hypertrophy is characterized by the parallel addition of sarcomeres leading to the lateral growth of individual cardiomyocytes. In contrast, volume overload, e.g., caused by aortic regurgitation, initiates eccentric hypertrophy to cope with the increase in wall stress during diastole. Eccentric hypertrophy is characterized by the addition of sarcomeres in series leading to increased cardiomyocyte length (Bernardo et al. 2010; Gajarsa and Kloner 2011).

I.VII Pathological cardiomyocyte hypertrophy and the fetal gene program  The partial recapitulation of the fetal cardiac gene program is illustrated by the following genes which show markedly different expression levels in the fetus as compared to the adult, and
which are often used as indicators of cardiac hypertrophy: ANF, MHCβ, MHCα, and SERCA2a. Atrial natriuretic factor (ANF) expression is highly expressed in the developing myocardium and levels decrease strongly after birth (Sergeeva and Christoffels 2013). In response to cardiac stress, Anf mRNA levels drastically increase and they are a consequently a widely used marker for the hypertrophic response (Sergeeva and Christoffels 2013; Sergeeva et al. 2014; Pikkarainen et al. 2003). In rodents, the re-expression of the fetal gene program also includes the shift in isoform expression of the cardiac contractile protein myosin heavy chain. Myosin heavy chain β (MHCβ, encoded by Myh7) is the predominant isoform in the developing rodent heart, but during early postnatal life it is gradually replaced by myosin heavy chain α (MHCα, encoded by Myh6) (Morkin 2000). Cardiac stress induces a shift back from the MHCα to the MHCβ isoform in the adult heart. Therefore, in rodent hearts a decrease in the α/β ratio is used as a marker for re-expression of the fetal gene program, and is associated with cardiac hypertrophy. It should be noted that the relative expression of MHCs in the human heart is different. Whereas MHCα predominates in non-failing adult rodent hearts, the adult human ventricle expresses approximately 95% MHCβ (Miyata et al. 2000; Reiser et al. 2001). Nevertheless, a further increase in the percentage MHCβ is also seen in human heart failure (Lowes et al. 1997). Although MHCβ is the predominant isoform in normal adult human hearts, it has been suggested that even small increases in MHCβ expression can significantly reduce cardiomyocyte power output (Herron and McDonald 2002). Sarcoplasmic reticulum Ca2+ ATPase 2a (SERCA2a) expression, normally expressed at higher levels in the adult than in the fetal ventricle, decreases in response to cardiac stress (Periasamy, Bhupathy, and Babu 2008). Diminished Ca2+ transport activity of the sarcoplasmic reticulum leads to a reduced release of Ca2+ during systole, which leads to reduction in force development. In addition, the slow rate of Ca2+ removal affects diastolic function (reviewed by Muller and Simonides 2005).

The relevance of the recapitulation of the fetal gene program in cardiac remodeling is a matter of debate. An adaptive role is suggested based on several observations. The shift to the energetically more efficient MHCβ isoform, as well the decreased expression of SERCA2a, will result in reduced energy turnover associated with contraction in the stressed myocardium (Bernardo et al. 2010; Dorn 2007). Nevertheless, recent studies suggest that the reduction of SERCA2a is a pivotal aspect of contractile dysfunction and failure (Wahlquist et al. 2014). In addition, only modest re-expression of the fetal gene program was observed in transgenic models of physiological hypertrophy [see also section I.IX] (Bernardo et al. 2010). The prevailing view is that the re-expression of the fetal gene program is a maladaptive response, also because the extent of the expression of this program is associated with the progressive decline in cardiac function in both animal models and man (Lowes et al. 2002; Rajabi et al. 2007).

I.VIII Signal transduction in pathological cardiomyocyte hypertrophy

MI-induced hypertrophy of the non-infarcted LV is considered to be essentially irreversible (Bernardo et al. 2010). While the phenotypical changes and consequences are clear, the underlying signal-transduction pathways are exceptionally complex and not fully resolved. Stress signals from various membrane receptors, including mechano-receptors, lead to the activation of downstream signaling cascades, among which cross talk exists. Animal studies have shown that distinct signaling pathways control the induction of pathological cardiac hypertrophy. The best-characterized pathway among these involves the G-protein coupled receptors (GPCRs) [Fig. 2]. In response to a pathological stimulus, neurohormones/vasoactive factors, such as ANGII, and NE and endothelin-1 (ET-1), are released. Binding of these ligands to GPCRs causes activation of downstream signaling proteins, including phospholipase C (PLC), protein kinase A (PKA) and C (PKC), calmodulin (CaM), and mitogen-activated protein kinases (MAPKs). Although the exact mechanism remains unclear, the MAPK pathway plays a central role in the regulation of
hypertrophy. Consecutive phosphorylation of different kinases leads to the activation of the three major MAPK pathway branches: p38-MAPK, c-jun N-terminal kinases (JNKs) and extracellular signal-regulated kinases (ERKs) (Bernardo et al. 2010). More is known about the calcium/calmodulin pathway, which activates calcineurin and calcium/calmodulin dependent protein kinases (CaMKs) (Bernardo et al. 2010). Once activated, calcineurin directly dephosphorylates members of the nuclear factor of activated T-cells (NFAT) transcription factor family in the cytoplasm, promoting their translocation into the nucleus (Molkentin 2004). Indeed, cardiac-specific overexpression of calcineurin or NFAT leads to significant cardiac hypertrophy that progresses to heart failure (Molkentin et al. 1998). Calmodulin-dependent CaMKII signaling is thought to exert its effect on pathological cardiac hypertrophy by causing phosphorylation of histone deacetylase 4 (HDAC4), which in turn relieves the repression of the transcription factor myocyte enhancer factor 2 (MEF2) (Passier et al. 2000). Like NFAT, MEF2 is able to activate pro-hypertrophic gene expression (Passier et al. 2000; Lu et al. 2000; Paquette et al. 2011; Molkentin 2004).

I.IX Signal transduction in physiological cardiomyocyte hypertrophy

In contrast to pathological cardiac hypertrophy, physiological cardiac hypertrophy is characterized by adaptive growth and maintenance of cardiac function, such as in postnatal development, pregnancy, chronic exercise training, and increased TH signaling (Bernardo et al. 2010; Heineke and Molkentin 2006). Also, this form of cardiomyocyte growth in the adult heart is fully reversible. Physiological cardiac hypertrophy involves activation of the insulin-like...
growth factor-1/phosphatidylinositol 3-kinase (IGF-1/PI3K) signaling pathway [Fig. 2]. Several animal studies have shown that IGF-1 and its receptor IGF-1R play a central role in signaling physiological growth of the heart (reviewed by Bernardo et al. 2010). A study using transgenic mice that overexpressed IGF-1R, reported that these mice developed cardiac hypertrophy with enhanced systolic function without detrimental effects on cardiac function. The hearts of mice overexpressing IGF-1R were enlarged and systolic function was enhanced, with no evidence of pathology (McMullen et al. 2004), whereas cardiomyocyte-specific ablation of the receptor blunted the hypertrophic response to exercise (J. Kim et al. 2008). Equally, inhibition of the downstream effector of IGF-1 signaling, PI3Kα, which is a heterodimer consisting of a p85 regulatory and a p110α catalytic subunit (Heineke and Molkentin 2006), attenuated the hypertrophic response to swim exercise. Furthermore, constitutive cardiac overexpression of PI3Kα induced physiological hypertrophy (Shioi et al. 2000). Interestingly, inhibition of PI3Kα did not affect the pathological hypertrophic response to aortic banding (McMullen et al. 2003). This illustrates that PI3Kα is essential for physiological cardiac hypertrophy and does not play a critical role in hypertrophy induced by pressure overload. Downstream of PI3Kα is Akt, which becomes activated upon phosphorylation. Activated Akt directly promotes protein synthesis by inhibiting the glycogen synthase kinase 3β (GSK-3β) repression of prohypertrophic transcription factors and enhances protein synthesis by activating mTOR signaling (Maillet, van Berlo, and Molkentin 2013; Bernardo et al. 2010). Although different cardiac phenotypes of Akt transgenic mice led to a debate about the role of this molecule in pathological or physiological cardiac hypertrophy, the prevailing view is that it is primarily involved in the latter (Bernardo et al. 2010). Other pathways that are indicated to play a role in physiological cardiac hypertrophy include JAK/STAT and TH signalling [see also section II.VII].

I.X MicroRNAs and heart failure

As mentioned in the previous sections, research of the past several decades has identified multiple triggers and signal transduction pathways modulating gene transcription contributing to the phenotypic changes that characterize pathological remodeling. Nevertheless, recent studies have shown an important role for short (~22 nucleotides) non-coding RNAs in the regulation of cardiac gene expression by virtue of their ability to induce degradation of specific target mRNAs or reduce the efficiency of translation (Thum, Catalucci, and Bauersachs 2008; van Rooij et al. 2006; Dirkx, da Costa Martins, and De Windt 2013; Bartel 2004). These so-called microRNAs (miRNAs) are individually produced from their own transcription units or are located within an intron of a mRNA. In addition, many miRNAs are grouped in clusters (Bartel 2004). Most of the transcribed miRNAs form a hairpin structure termed primary, or pri-miRNA (Bartel 2004; Y. Lee 2002). In the nucleus the pri-miRNA is processed into a ~70 nucleotides long pre-miRNA (Yoontae Lee et al. 2003; Y. Wang et al. 2007). Correctly processed pre-miRNAs are exported from the nucleus to the cytoplasm (Yi et al. 2003; Lund et al. 2004) and are incorporated into the RNA-induced silencing complex (RISC). This complex binds to the 3’ untranslated region (UTR) of its mRNA target through base pairing between the miRNA seed region (position 2 to 8 from the 5’ end) and complementary sequences in the UTR (Bartel 2004). Depending on the degree of complementarity between the miRNA seed region and the mRNA target sequence, the binding of the miRNA results in mRNA degradation or translational repression of the target mRNA (Bartel 2009). Although multiple different miRNAs are thought to be required to fine-tune expression of a particular target gene, several studies have provided insight in the critical roles of single miRNAs in the different processes associated with development and disease, and in particular the progression of heart failure [Fig. 2]. For example, miRNA profiling studies have shown that the expression of miR-214 is upregulated in multiple animal models and patients with end-stage heart failure (da Costa Martins et al. 2010; van Rooij et al. 2006; van Rooij et al. 2008). Additional
studies, both in cell culture and animal models, indicated a critical role of this miRNA in cardiomyocyte hypertrophy (van Rooij et al., 2006; el Azzouzi et al., 2013). In a recent study, Wahlquist et al. showed a similarly unique role of miR-25 in the reduction of SERCA2a levels in pathological hypertrophy. They reported that inhibition of miR-25 attenuated or even reversed established heart failure at least in part by increasing SERCA2a expression levels in mice (Wahlquist et al. 2014).

I.XI Cardiac regeneration The process of cardiomyocyte hypertrophy, rather than hyperplasia, in response to chronically increased cardiac load exemplifies the lack of regenerative capability of the myocardium. Cardiomyocytes withdraw from the cell cycle shortly after birth. At this point myocardial growth shifts from hyperplastic to hypertrophic, and adult cardiomyocytes are considered to be terminally differentiated cells. Consequently, the focus has been on the increase in cardiac mass and the signaling pathways involved in cardiac hypertrophy (Molkentin and Dorn 2001; Bernardo et al. 2010). Nevertheless, experiments with zebra fish models demonstrated that surgical removal of 20% of the ventricle resulted in complete cardiac regeneration devoid of scar tissue (Jopling et al. 2010), mediated by the de-differentiation and proliferation of pre-existing cardiomyocytes and not by the mobilization of undifferentiated precursor cells (Jopling, Boue, and Belmonte 2011; Kikuchi et al. 2010). Even though the mammalian heart appears to lack the remarkable regenerative capacity of the zebra fish, recent mouse studies suggest that a similar process of cardiac regeneration may be present during a brief postnatal period (Porrello, Mahmoud, et al. 2011; Naqvi et al. 2014). Evidence has emerged indicating that a limited capacity for cardiac renewal also occurs in the human heart, although the cardiomyocyte source remains a matter of debate. The seminal observation in this field was the analysis of cardiomyocyte turnover, using integration into the genome of $^{14}$C generated by nuclear bomb tests during the Cold War (Bergmann et al. 2009). A turnover of 1% per year at the age of 25 was observed, decreasing to 0.45% per year at the age of 75, indicating that up to 50% of cardiomyocytes are exchanged during life. Obviously the capacity to renew cells is not sufficient to cope with the substantial loss of cardiomyocytes following MI.

The heart is a major target organ of TH and marked similarities in gene expression in the absence of TH (hypothyroidism) and heart failure suggest a role for reduced TH action in the etiology of cardiac dysfunction. In the following sections we summarize the basic aspects of TH metabolism and action, as well as the current evidence supporting the suggested hypothyroid condition of the failing heart.
THs are involved in development and in maintaining proper metabolic homeostasis of virtually all mammalian tissues as is illustrated by the wide range of clinical symptoms associated with reduced as well as excess levels of THs (reviewed by Klein and Ojamaa 2001). As part of the endocrine system, the hypothalamus-pituitary-thyroid (HPT) axis regulates TH levels. The thyroid gland primarily secretes the inactive pro-hormone thyroxin (T4; 3,5,3’5’-tetraiodothyronine), and in small amounts the active form triiodothyronine (T3; 3,5,3’-triiodothyronine) in response to the thyroid-stimulating hormone thyrotrophin (TSH). The release of TSH, which is produced by the pituitary, is stimulated by the hypothalamic hormone thyrotrophin-releasing hormone (TRH). In turn, circulating T4 and T3 exert a negative feedback on the release of TSH and TRH, maintaining TH levels within a narrow range (Zoeller, Tan, and Tyl 2007). Enzymatic inactivation of T4 and T3 gives rise to reverse T3 (rT3; 3,3’,5’-triiodothyronine) and T2 (3,3’-diiodothyronine), respectively (see below).

II.I Deiodination of thyroid hormones

The activation and inactivation of THs depends on the removal of a distinct iodine residue from either the phenolic outer ring, or the inner tyrosyl ring. This conversion is catalyzed by a specialized group of selenoproteins, called deiodinases. Three types have been identified, i.e., type I, type II, and type III deiodinase (DIO1, DIO2, and DIO3, respectively) (reviewed by Gereben et al. 2008). These enzymes share the presence of the rare amino acid selenocysteine (Sec) within the conserved active center of the protein [see also section III.V]. However, they differ in their catalytic properties, tissue distribution and developmental expression [Fig. 3].

The conversion of T4 to T3 in peripheral organs, e.g. liver, adipose tissue, and, to some extent, skeletal muscle accounts for the largest part of T3 that is produced. This step is catalyzed by DIO1 and DIO2, and entails the removal of a single iodine atom from the outer ring of T4. DIO1, located in the plasma membrane, is primarily expressed in the liver and kidney. However, transcripts of DIO1 are also identified in the thyroid, pituitary gland, intestine, placenta, and gonads. DIO1 is the only deiodinase capable of both outer- and inner-ring deiodination. Although the exact role of DIO1 in human TH metabolism is not fully understood, it is thought that DIO1 is responsible for providing at least part of the circulating T3. However the preference of DIO1 for rT3 as substrate for the outer-ring deiodination, instead of T4, suggests that DIO1 plays a role in clearing and recycling iodine within the system (reviewed by Maia et al. 2011). DIO2, located in the endoplasmic reticulum, also contributes to the production of plasma T3, but its primary role appears to lie in generating T3 in tissues in which DIO2 is expressed. Transcripts are found in a variety of tissues, including brown adipose tissue (BAT), brain, pituitary, and, at low levels, also in human heart and skeletal muscle. In BAT, cold-induced sympathetic activation and stimulation of β-adrenergic receptors is the driving force behind the local increase in DIO2 expression. The accelerated conversion of T4 to T3 results in the expression of the T3-responsive mitochondrial uncoupling protein 1 (UCP-1), the principal thermogenic protein (reviewed by Bianco and Kim 2006). The regulated expression of DIO2 in BAT is an example of the emerging general concept in which regulation of deiodinase activities is used to increase or decrease T3 signaling in a cell specific way.

The third deiodinase, DIO3, catalyzes the inactivation of both T4 and T3, producing the inactive THs rT3 and T2, respectively (reviewed by (Gereben et al. 2008). This deiodinase is highly expressed in the placenta and in most fetal tissues [see also section II.IV]. In the adult, DIO3 expression is virtually absent in most tissues, except for skin, and a variety of cells in the brain. DIO3 is primarily located in the plasma membrane, and
although extracellular activity has been proposed, intracellular THs appear to be the principle substrate for DIO3 (reviewed by Pol, Muller, and Simonides 2010). This is in line with the reduction of local TH levels observed when DIO3 is re-expressed in the adult heart after injury (Pol et al. 2011) [see also Introduction Part III.a].

II.II TH action

TH primarily acts at the level of gene regulation in virtually all tissues and this is mediated by nuclear TH receptors (TR). Because of the supposed lipophilic nature of THs, uptake into cells was long thought to be a passive process. However, following the seminal work of Visser et al. (Visser et al. 2008; Friesema et al. 2003) uptake is now known to require TH-transporters. Several transports have been identified, of which monocarboxylate transporter 8 (MCT8), MCT10 and organic anion-transporting polypeptide 1C1 (OATP1C1) have high affinity for THs (Friesema et al. 2008; Danzi and Klein 2014; M. Li et al. 2014; Visser et al. 2008). Of these transporters, MCT10 has the highest affinity for the biologically relevant TH-molecule, T3 (Friesema et al. 2008).

Expression of MCT8 and MCT10 has been detected in numerous tissues, including brain, liver, kidney and heart (Bonen, Heynen, & Hatta, 2006; Friesema et al., 2003; Schutkowski, Wege, Stangl, & König, 2014). The relative contribution of MCT8 and MCT10 to TH uptake in heart is unknown. However, earlier studies describing active uptake of TH in isolated neonatal rat cardiomyocytes and the embryonic heart cell line H9C2 found that, under physiological conditions, cardiomyocytes take up TH with a distinct preference for T3 over T4. (Everts et al., 1996; van der Putten, 2001, 2002). This may indicate that MCT10 is the predominant transporter in cardiomyocytes. Selective uptake of T3 is also supported by in vivo studies. Analyses of transcription of the TH-regulated gene Myh6 in the rodent heart showed that already within 30 minutes after T3 administration, the level of its unspliced RNA transcript increased. Although increasing Myh6 RNA levels were also observed after T4 administration, this was delayed by almost 12 hours, coinciding with rising serum T3 levels (Danzi, Ojamaa, & Klein, 2003).

T3 binds to TRs, encoded by two genes: TRα and TRβ. Both produce three isoforms as a result of alternative splicing. The expression of the different isoforms is tissue specific and is developmentally regulated. TRα produces one T3-binding isoform (TRα-1) and two non-T3-binding isoforms (TRα-2 and TRα-3). While TRα-1 is already expressed during early development, TRβ isoforms 1, 2, and 3 (all T3-binding isoforms) are expressed toward the final stage of development in various tissues (reviewed by
The heart predominantly expresses TRα-1 (Constantinos Pantos, Mourouzis, et al. 2007). TRs are DNA-binding transcription factors that recognize specific DNA sequences located within upstream promoter regions of TH-regulated genes, called TH responsive elements (TREs). TREs consist of multiple copies of the consensus sequence (A/G)GGT(C/A/G)A. This so-called ‘half-site’ binding motif can be arranged as palindromes, direct repeats, or inverted repeats. TRs can bind TREs as homodimers, but typically TRs heterodimerize with other receptors. The retinoic X receptor (RXR) is one of the preferred heterodimerization partners for TRs (Cheng, Leonard, and Davis 2010). This complex increases the binding of TRs to TREs as well as the transcriptional activation of TH-regulated genes (Bassett, Harvey, and Williams 2003; Kahaly and Dillmann 2005). In addition to the differential expression of TR isoforms, the diversity of TREs, and the availability of heterodimerization partners, the level of transcriptional activity is also regulated by co-repressors and co-activators. In the absence of T3, the TR-RXR heterodimer binds to a TRE and interacts with a co-repressor complex, which represses transcription of positively regulated genes. This co-repressor complex includes nuclear receptor co-repressor (N-CoR) and silencing mediator of retinoic and thyroid hormone receptors (SMRT). These proteins interact with histone deacetylases (HDACs) to form large repression complexes. Binding of T3 results in displacement of co-repressors and the recruitment of a co-activator complex, which includes histone acetyltransferases (HATs). Histone acetylation unfolds the chromatin structure and induces transcription (Cheng, Leonard, and Yen 2001; Jepsen and Rosenfeld 2002; Yoh and Privalsky 2001; Astapova et al. 2008). The underlying mechanisms of negative regulation by liganded TRs remain largely unknown.

In addition to the classical TR-complex-mediated genomic activity, TH also exerts rapid non-genomic actions at the level of the plasma membrane, or through TRs located within the cytoplasm. The plasma membrane protein integrin αvβ3 has been shown to contain a binding domain for THs, primarily T4. From this site the TH-signal is transduced mainly via downstream targets of MAPK, such as extracellular signal-regulated kinases (ERK1/2). Activation of downstream cascades affects transcription activity. Furthermore, it has been shown that T3-ligated TRα1, located within the cytoplasm, binds the p85α regulatory subunit of PI3K. Activation of the PI3K/Akt pathway may underlie the mechanism via which TH initiates physiological hypertrophy [see also section I.VIII] (reviewed by Cheng, Leonard, and Davis 2010; Bernardo et al. 2010; P. J. Davis, Leonard, and Davis 2008; Oetting and Yen 2007; Bassett, Harvey, and Williams 2003).

II.III TH and microRNAs

Considering the importance of TH in the regulation of numerous processes in a variety of tissues, it is remarkable that only a handful of miRNAs have so far been linked to TH-action. Visser et al. examined the effects of T4 on the human skeletal muscle transcriptome. Microarray analysis of transcript levels using biopsies from euthyroid and hypothyroid patients showed a marked downregulation of miRNAs miR-206 and 133b upon T4 treatment of these patients (Visser et al. 2009). Dong et al. supported these results by studies on miRNAs expressed in liver of hypothyroid mice. They found a significant upregulation of miR-206/133b when mice were depleted of TH. In addition, they found significant upregulation of the closely homologous miR-1-1/133a-2 (Dong et al. 2010). In human hyperthyroid skeletal muscle biopsies the latter miRNAs were also found to be differentially expressed, but to a lesser extent (Visser et al. 2009). By far the most extensively studied TH-regulated miRNAs are encoded within introns of the MHC-isoforms Myh6 and Myh7, miR-208a and miR-208b respectively. These MHC isoforms are classic examples of genes that are transcriptionally stimulated (Myh6) and inhibited (Myh7) by TH. Treating isolated rat cardiomyocytes with TH revealed that expression of miR-208a was induced while miR-208b expression levels were reduced. This suggested that the intronic miRNAs and their
host genes are co-expressed and regulated by a common regulator, TH (Callis et al. 2009; Diniz, Takano, and Barreto-Chaves 2013) [see also section I.IX].

II.IV TH levels and deiodinase activity during fetal development

It has been shown that tissue exposure to THs during development is stringently regulated both spatially and temporally via deiodinase activity (Hernandez et al. 2006). Of the three deiodinases, evidence suggests that only DIO2 and DIO3 play a major role in development. DIO3 activity is found to be much higher in the placenta and developing tissues than in mature tissues. Expression is already observed in several tissues before the onset of fetal thyroid function. This suggests that DIO3 plays a major role in protecting the fetus from exposure to excessive or ‘adult’ levels of TH (reviewed by Galton 2005; Bianco and Kim 2006). The importance of DIO3 activity during development became clear by evaluating DIO3 knock-out (D3KO) mice. It was shown that these mice had several abnormalities, including elevated T3 plasma levels, growth retardation, increased expression of T3-regulated genes in the brain, impaired maturation of the HPT-axis, and impaired hearing (Ng et al. 2009; Hernandez et al. 2006). Around birth DIO3 expression is turned off in virtually every tissue and following the sharp rise in plasma T3 levels after the first week of postnatal life, TH-signaling increases substantially. In heart as well as skeletal muscle, this surge in T3 activity is involved in driving the fetal-to adult switch in gene expression [see also section I.VII].

In contrast to the high DIO3 activity during development, DIO2 expression is more restricted. It is suggested that short bursts of DIO2 activity are needed to provide an effective concentration of T3 at a precise time point during the development of a particular tissue (reviewed by Galton 2005; Bianco and Kim 2006). Most of the T3 that is produced within the cell remains there (Bianco et al. 2002). Several observations that have been obtained in rodents pointed out the importance of the spatially and temporally regulated DIO2 activity in neuronal development, in particular the auditory system. Reduced T3 levels in the developing ear of DIO2 knock-out mice resulted in suppressed cochlea differentiation, which is in contrast to the premature acceleration observed in D3KO mice (Ng et al. 2009; Ng et al. 2004). This indicates that a precisely timed activation of either of the deiodinases, and associated change in the level of THs, during development is of great importance.

II.V The role of TH in proliferation and differentiation

Genes involved in cell cycle control or cellular differentiation, i.e., E2F1, p53, c-Myc, and cyclin D1, can be directly regulated by T3 or indirectly via T3-dependent modulation of the activity of different signaling pathways (reviewed by Pascual and Aranda 2013; Kress, Samarut, and Plateroti 2009). Thus, it is essential to maintain TH levels at a precisely regulated level during fetal and neonatal life to support appropriate cell proliferation and differentiation (Hernandez et al. 2006). Whether proliferation or differentiation is initiated depends on cell type, cell context (physiological vs pathological), and developmental status (reviewed by Pascual and Aranda 2013; Kress, Samarut, and Plateroti 2009; Bianco et al. 2002). However, in general T3 is considered to promote differentiation and attenuate proliferation. For example, in the developing ovine heart it was shown that during the first half of gestation, when TH levels were low, fetal cardiomyocytes proliferate. At two-thirds of gestation, due to the maturation of fetal HPT-axis, the increase of T3 initiated cardiomyocyte differentiation/maturation and decreased their proliferative capabilities. This is in line with a study where premature elevation of T3 in fetal sheep resulted in a ‘mature’ cardiac phenotype (Chattergoon, Giraud, and Thornburg 2007). In rodents, the fetal thyroid gland starts to secrete TH from 3-5 days before birth (reviewed by M. Li et al. 2014). TH levels gradually increase with an initial peak around postnatal day 7 (Howdeshell 2002). It was only recent that a more marked rise in T3 was observed at postnatal day 12-15, which, although counterintuitive, was found to be associated with
a cardiac hyperplastic burst between postnatal day 11 and 18, adding 500,000 cardiomyocytes to the preadolescent mouse heart. Naqvi et al. reported a T3-dependent increase in IGF-1 expression at postnatal day 15 relative to postnatal day 10 (Naqvi et al. 2014). It is known that IGF-1 causes fetal cardiomyocyte proliferation by activating the Akt/PI3K pathway (Sundgren et al. 2003). While constitutively activated Akt results in cardiac hypertrophy, nuclear localization of Akt increases cardiomyocyte numbers (Condorelli et al. 2002; Rota et al. 2005). At postnatal day 15, Akt was mostly located within the cardiomyocyte nucleus, which supports a permissive role for T3 in postnatal cardiomyocyte proliferation (Naqvi et al. 2014). Furthermore, the proliferation of existing cardiomyocytes was prevented by inhibiting T3-synthesis and subsequent IGF-1 expression (Naqvi et al. 2014). In addition to earlier findings, which suggested mouse cardiomyocytes are not able to divide after postnatal day 7 (Porrello, Mahmoud, et al. 2011), Naqvi et al. concluded that cardiomyocytes of rodents maintain their proliferative capabilities until they are terminally differentiated at approximately postnatal day 20 (Naqvi et al. 2014).

II.VI TH-effects in adult tissue

Besides the involvement in processes essential for normal growth and development, TH is involved in regulating metabolism in the adult (Cheng, Leonard, and Davis 2010). The stimulatory effects of THs on whole body metabolic activity have been recognized for over a century and involve both energy consumption and substrate metabolism generating ATP (reviewed by Hulbert 2000). Both direct and indirect actions of TH contribute to these effects. An example of the former is the stimulatory effect of TH on skeletal muscle contractility, which involves a shift in MHC isoforms and an increase in SERCA activity, similar to its action in the heart. Because skeletal muscle comprises 30 to 40% of the mass in the human body, the TH-dependent increase in the energy cost of contraction contributes significantly to the body’s higher energy turnover (Simonides and van Hardeveld 2008). TH has been shown to be involved in adult tissue homeostasis, regeneration and repair. For example, skeletal muscle consists of postmitotic cells that do not divide. Nonetheless, skeletal muscle tissue exhibits the capacity to regenerate lost tissue through the activation of satellite cells. Myogenesis, i.e., the proliferation and subsequent fusion and differentiation of satellite cells, is under strict control of the T3-dependent master regulator of myogenic developmental and regeneration program, MYOD1. DIO2 and DIO3 are expressed in skeletal muscle of both man and rodents (reviewed by Domenico Salvatore et al. 2013). DIO2 expression is elevated in differentiating myoblasts and muscle precursor cells (MPCs). Loss of DIO2 impairs precursor differentiation, as it reduces the levels of intracellular T3 and consequently MYOD1 expression. The differentiation defect as is seen in DIO2-deficient MPCs was coupled to an increase in the proliferative capacity of these cells, suggesting that a reduction in the levels of intracellular produced T3 enhances the proliferative capacity of the MPCs in vivo (Dentice et al. 2010). This is in line with a study that observed increased DIO3 levels in proliferating MPCs, and suppressed levels in differentiated MPCs (Dentice et al. 2014). On the other hand, the absence of DIO3 in activated MPCs caused cell death due to excessive levels of intracellular T3 (Dentice et al. 2014). Sequential expression of DIO3 followed by DIO2 in activated MPCs could balance the different TH requirements during the cell lineage progression with DIO3-mediated low intracellular T3 allowing for the expansion of the satellite cell pool, whereas increases in DIO2 expression and activity facilitate MPC differentiation (Domenico Salvatore et al. 2013).

II.VII TH and the cardiac phenotype

The influence of TH-signaling on development, growth and function is clearly shown in one of the principal target organ of T3, the heart. In rodents, the gradual increase in TH signaling after birth induces a transcriptional switch that leads to the characteristic gene expression that is observed in the adult
heart. A well-described example is the switch in MHC isoforms. The expression of the positively regulated Myh6 almost completely replaces the negatively regulated Myh7 within seven days after birth (reviewed by Maillet, van Berlo, and Molkentin 2013). Other characteristic examples of cardiac TH-regulated genes are the positively regulated sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (Serca2a), and the voltage-gated potassium channels (Kv1.5, Kv4.2, Kv4.3). Similar to Myh7, the SERCA2a inhibitor phospholamban is an example of a gene which is negatively regulated by TH (Klein and Ojamaa 2001; Danzi and Klein 2002). The effects of TH on cardiac gene expression are reflected by the significant changes in cardiovascular function as a result of thyroid dysfunction. Cardiac output increases by 50-300% in hyperthyroidism relative to normal individuals as a result of increased heart rate and enhanced systolic and diastolic function, i.e., increased ejection fraction. A decrease in systemic vascular resistance induced by TH decreases afterload and improves cardiac efficiency. In addition, the increase in blood volume results in increased venous return and preload, further enhancing cardiac output (reviewed by Klein and Ojamaa 2001; Danzi and Klein 2014). Apart from the above-described effects on the expression of key proteins that determine cardiac function, increasing T3 levels also induce cardiac hypertrophy [Fig. 2]. This form of hypertrophy is considered physiological as it is not associated with adverse remodeling, impairment of contractility or fibrosis (Ching et al. 1996; Klein and Ojamaa 2001). Acting through cytosolic TRα, T3 can activate the PI3K-Akt pathway involved in protein synthesis and cellular growth (Kuzman et al. 2005; Kuzman, O’Connell, and Gerdes 2007; Kenessey and Ojamaa 2006), but the principal trigger for hypertrophy is the T3-induced increase in hemodynamic load of the heart. This was demonstrated in a rat model with infrarena\[\]l heterotopic cardiac isografts. Here, TH administration resulted in a significant increase in total heart weight of the in situ heart, whereas the heart weight, rate of protein synthesis and total myosin content of the heterotopic heart, i.e., non-working heart remained unchanged (Klein and Hong 1986; Shao et al. 2000). However, all T3-induced changes in gene expression, such as the MHC isoform shift, were the same in the in situ and heterotopic heart.

Changes observed in patients with hypothyroidism are the opposite of those seen in hyperthyroidism. They include a reduction of contractility, heart rate and blood volume as well as an increase in systemic vascular resistance, culminating in a 30-50% reduction in cardiac output relative to normal individuals. As a consequence of the increased afterload, stroke volume and cardiac output are reduced (Klein and Ojamaa 2001). The changes in gene expression that underlie the cardiac-specific responses to hypothyroidism bear resemblance to the heart failure phenotype.
As already mentioned at the conclusion of Part I and II, involvement of impaired TH signaling in cardiac pathological remodeling is suggested by similar changes in the expression of some of the TH-regulated cardiac genes in hypothyroidism and in heart failure. Characteristic examples of changes with important consequences for contractility are the reduction of *Myh6* and *Serca2a* expression, and the increased expression of *Myh7* (Klein and Ojamaa 2001). Ojamaa et al. demonstrated, in the setting of myocardial infarction (MI), alterations in specific TH-responsive genes in the rat (Ojamaa et al. 2000).

A decrease in TH signaling can be the result of limited availability of TH or changes in nuclear TR and/or co-factor expression. The situation is complicated by the fact that unliganded TRα-1 suppresses TH-activated genes, and stimulates TH-inhibited genes. Therefore, decreased TH signaling may also result from excess nuclear TRα-1 levels, increasing the ratio of unliganded over liganded TR in the nucleus. However, the available evidence indicates that a reduction in expression of TRα-1, rather than an increase, may play a role in impaired TH signaling in heart failure. Following MI, TRα-1 levels were shown to be reduced in mice (Mourouzis et al. 2013), and a study using a mouse model of LV pressure overload showed that cardiac function improved after TR expression was increased using viral transduction (Belke et al. 2007). Nevertheless, the role of TRs in cardiac remodeling remains a matter of debate with both increased and decreased TR expression being suggested.

Indication for a local inactivation of T3 in the failing heart as a possible cause of a hypothyroid condition was observed for the first time in a rat model of right ventricular (RV) hypertrophy induced by pulmonary arterial hypertension (PAH) (Wassen et al. 2002). In this model the increase in RV afterload induced pathological hypertrophy of the right ventricle. When compared to LV, the degree of RV hypertrophy was associated with pronounced changes in gene expression involved in pathological hypertrophy, such as the reduction of *Serca2a* mRNA levels and the switch from *Myh6* to *Myh7* isoform (Buermans et al. 2005; Wassen et al. 2002; Simonides et al. 2008). Interestingly, these changes were accompanied by a strong induction of DIO3 activity in the remodeled RV compared to the LV, where DIO3 activity remained at the low level seen in both the RV and LV of control animals (Simonides et al. 2008; Wassen et al. 2002). Moreover, the level of RV DIO3 activity correlated with the severity of RV failure (Wassen et al. 2002). Induction of DIO3 activity was also found in a mouse model of chronic pressure overload of the LV due aortic stenosis (Trivieri et al. 2006) and diabetes mellitus related cardiomyopathy (Weltman et al. 2014). Additional research using a rat model of MI observed identical levels of DIO3 activity in the infarcted LV 1 week post-surgery to those shown in the RV of PAH rats (Olivares et al. 2007; Wassen et al. 2002). A more recent study, using a mouse model of MI, showed an induction already at 1 week post-MI surgery that remained at least until 8 weeks after MI (Pol et al. 2011). The re-expression of DIO3 in the mouse heart following MI is seen in an estimated 20% of cardiomyocytes throughout the spared, remodeling myocardium. In both the PAH model and the model for post-MI LV remodeling the increase in DIO3 activity was associated with a ventricle-specific ~50% reduction in tissue T3 levels.

The reduction of tissue T3 levels in the failing RV or LV in these studies could not be explained by reduction of plasma T3 levels, often seen in severe illness, including advanced heart failure. Importantly, *in vivo* determination of cardiac T3-dependent transcription in the failing ventricle in both models conclusively showed a cardiomyocyte-specific reduction, to a level similar to overt hypothyroidism (Simonides et al. 2008; Pol et al. 2011). Although a contribution to this effect of altered levels of TR or its co-factors cannot be excluded, the increase in cardiac DIO3 activity and associated
III.I Regulation of DIO3 expression in the heart

As described above, increased DIO3 activity and the associated decrease in local T3 levels have been studied in a variety of animal models. The re-expression of DIO3 may be accounted for by at least three different pathways, which are also indicated to be involved in LV remodeling (reviewed by Pol, Muller, and Simonides 2010). A study by Huang et al. showed that transforming growth factor β (TGFβ) and MAPK signaling induce DIO3 expression, via p38, SMAD, and ERK, in diverse human cell types, including fetal and adult fibroblasts, fetal epithelia, and skeletal muscle myoblasts (S. A. Huang et al. 2005). Furthermore, both TGFβ and MAPK signaling are known for their involvement in ventricular hypertrophy and cardiac remodeling (Lim and Zhu 2006; Rosenkranz 2004; Bujak and Frangogiannis 2007; Muslin 2008). In models of MI it has been reported that TGFβ expression is increased, especially in the infarct and the surrounding border zone (reviewed by Bujak and Frangogiannis 2007).

In addition to TGFβ and MAPK signaling, it was shown that DIO3 is also directly regulated via sonic hedgehog (SHH) signaling. By analyzing keratinocytes isolated from basal cell carcinomas, Dentice et al. showed that SHH directly induces DIO3 transcription, which caused local TH levels to decrease. In addition, the local hypothyroidism in these cells was associated with the increased expression of cell cycle gene Cyclin D1 and proliferation. Equally, DIO3 overexpression in basal cell carcinoma has been shown to enhance proliferation of normal and malignant keratinocytes (Dentice et al. 2007). In the adult heart SHH signaling in cardiomyocytes and perivascular smooth muscle cells plays an important role in maintaining cardiac function. Furthermore, endogenous SHH expression has been shown to be upregulated after MI surgery in mice (Kusano et al. 2005). Thus far, the involvement of SHH signaling in regulating cardiac DIO3 expression in the setting of post-MI induced LV remodeling has not been studied in detail.

In vitro analysis showed that under hypoxic conditions the hypoxia-inducible factor-1α (HIF-1α) interacts with the promoter region of DIO3 resulting in increased DIO3 expression levels and activity (Simonides et al. 2008). Under normoxic conditions, HIF-1α protein is ubiquitinated and then degraded. When oxygen demand exceeds supply, HIF-1α is no longer degraded and is then transported to the nucleus. Here it activates the transcription of hypoxia-responsive genes. Increased DIO3 expression and activity was confirmed in neurons, choriocarcinoma cells, hepatocytes, and isolated neonatal cardiomyocytes, while neither endometrial cells nor fibroblasts responded (Simonides et al. 2008). In the process of post-MI cardiac remodeling, which includes the increase in cross sectional area of stressed cardiomyocytes and development of interstitial fibrosis [see also section I.V], oxygen supply can be impeded due to decreasing capillary density and increasing oxygen diffusion distances (Des Tombe et al. 2002). Increased HIF-1α levels have been reported in various rodent models of MI within the first 24 hours after surgery (Kido et al. 2005). A key study, in which the effects of HIF-1α induction on DIO3 expression in the process of RV remodeling was analyzed in a model of pulmonary hypertension [see also Introduction Part III.A], found an RV-specific association between DIO3 activity and HIF-1α protein levels (Simonides et al. 2008).

Although the signaling cascades involving the above mentioned factors are all implicated in cardiac remodeling, none of these have as yet been conclusively shown to be involved in the re-expression of DIO3 in the failing heart. It is important to mention here that the heterogeneous expression of DIO3 throughout the myocardium implies activation of specific signaling cascades is some cells, but not in others. The resulting dilution when analyzing whole ventricle homogenates most likely explains the difficulty in identifying DIO3-regulating pathways.
III.II Is DIO3 activity adaptive or maladaptive? Whether this re-expression of DIO3 and the ensuing local hypothyroid condition is part of an adaptive response of the stressed myocardium aimed at reducing cellular metabolism or is involved in the pathological signaling causing heart failure is still a matter of debate. As described above, HIF-1α signaling is activated in those cells that are affected by the misbalance between oxygen supply and demand and the down-stream effects of HIF-1α are generally adaptive. These cells would benefit from a reduction in TH-dependent energy turnover and expression of DIO3 would therefore fit the adaptive response. Recent studies using a DIO3 knockout mouse model presented evidence that induction of cardiac DIO3 in response to a pathological hypertrophic stimulus, i.e., isoproterenol, is indeed adaptive. Here, the absence of DIO3 led to a further decrease in cardiac function when compared to isoproterenol-treated wild type mice, resulting in congestive heart failure and increased mortality. Of note, the results are inconclusive since these mice already developed a cardiac phenotype including fibrosis (Ueta et al. 2012).

DIO3 activity may also be maladaptive, since the local reduction of T3 will further impair cardiac function via dysregulation of TH-regulated genes involved in cardiac contractility. This includes the shift towards the slow contracting MHCβ, but importantly also the reduction of SERCA2a expression and the consequential dysregulation of Ca²⁺ homeostasis. Impaired SERCA2a activity is observed during later stages of cardiac remodeling and is associated with the transition from compensated hypertrophy to heart failure (reviewed by (Muller and Simonides 2005). Accordingly, restoring SERCA2A levels using gene transfer and inhibition of miR-25 [see also section I.X] improved cardiac function of heart failure in animal models (Wahlquist et al. 2014; Kawase et al. 2008).

Based on the time-course analysis of DIO3 expression in models of RV hypertrophy and failure (Buermans et al. 2005), as well the post-MI model of LV failure, Pol et al. concluded that the timing and extent of DIO3 induction most likely determines whether the resulting change in TH metabolism is adaptive or maladaptive (Pol, Muller, and Simonides 2010). In the context of post-MI remodeling, the persistent DIO3 activity is thought to contribute to the impairment of ventricular function.

III.III Involvement of DIO3 in tissue repair Besides the possibly adaptive effect of DIO3 on energy turnover in the stressed cardiomyocyte, DIO3 might also play a role in tissue repair. As was mentioned earlier, DIO3 is expressed in virtual every fetal tissue. As a consequence, these growing tissues are considered to be low in TH levels, which generally favors cell proliferation. As mentioned earlier, the regenerative capacity of the heart is exceptionally small, but it cannot be ruled out that increased levels of DIO3 in the injured heart, as part of the re-expression of the fetal gene program, are in fact linked to a proliferative drive. Precisely such a role of DIO3 has been documented in a variety of malignancies. DIO3 is induced in some malignant cell lines (Kester et al. 2006) and in a number of human tumors, i.e., oligodendromas, astrocytomas, gliomas, glioblastoma multiforme, TSH-secreting pituitary adenomas, colon adenomas, and carcinomas (reviewed by Luongo et al. 2013; Kress, Samarut, and Plateroti 2009). DIO3 overexpression in basal cell carcinoma has been shown to enhance proliferation of normal and malignant keratinocytes. Furthermore, it has been suggested that the balance between DIO3 and DIO2, i.e., fine-tuning T3 levels, is critical in modulating the balance between proliferation and differentiation of keratinocytes (Dentice et al. 2007).

The previous sections have highlighted the role of T3 in cardiac physiology and have shown that the induction of DIO3 activity and the associated local hypothyroidism is a common denominator in ventricular remodeling and the development of heart failure in various models. In the following section we take a closer look at DIO3.
DIO3 is located at the most distal part of the DLK1-DIO3 genomic imprinted region on mouse chromosome 12F1 (human chromosome 14q32), which includes, next to DIO3, the protein coding genes delta-like homologue 1 (DLK1) and Retrotransposon-like gene 1 (RTL1), as well as the non-coding RNA sequences Gtl2 (MEG3), Rian (MEG8), MIRG, and numerous miRNAs. Deletion studies in mice and mutations in this region in humans show a range of severe phenotypes, including growth deficiencies and developmental defects in the embryo and placenta, defects in adult metabolism and brain function (reviewed by da Rocha et al. 2008). The paternal chromosome expresses the coding genes DLK1, RT11, and DIO3, whereas the miRNAs and non-coding genes, GTL2, RIAN, and MIRG originate from the maternal chromosome. The miRNA-cluster located within the Dlk1-Dio3 genomic imprinted region is the largest currently known miRNA-cluster. In mouse, these miRNAs are mostly expressed as products of the non-coding transcripts Rtl1 antisense (Rtl1as) and Mirg (Seitz et al. 2004). Overexpression of these miRNAs has been associated with increased proliferative activity and disease progression, while others have shown tumor suppressive properties (Benetatos, Voulgaris, and Vartholomatos 2012). In hepatocellular carcinoma the upregulated miRNA signature has been positively correlated with stem cell markers (Luk et al. 2011).

DIO3 belongs to the family of 25 selenocysteine (Sec)-containing proteins (selenoproteins) (Kryukov et al. 2003), and is found in all vertebrates (Mariotti et al. 2012). A codon for Sec, UGA, is localized within the coding sequence of DIO3. This codon is in the vast majority of mRNAs recognized as a stop codon. The incorporation of Sec requires secondary structures to ensure translation is not terminated at this codon (Low 1996). The cis-acting sequences and trans-acting factors necessary for recoding the UGA codon have been well studied. The cis-acting sequences include the UGA codon itself and a mRNA stem loop structure located within the 3’UTR, the Sec-insertion-sequence (SECIS). This secondary structure in some cases can be located up to several kilobases away from the UGA codon. Only together with a complex containing factors such as Sec synthase (SelA), SECIS-binding-protein (SBP2), elongation factor (EFSec), and its cognate tRNA Sec, the UGA codon is recoded to recognize Sec instead (Berry 2005; Kuiper, Klootwijk, and Visser 2003; Low 1996; Köhrle et al. 2005; Bianco et al. 2002) [Fig. 4]. Although there is low homology between SECIS-elements, their secondary structures are highly conserved and contain consensus sequences that are crucial for the incorporation of Sec (Papp et al. 2007). The critical role of the SECIS-element was revealed by mutation of the conserved sites within the element, which completely impaired UGA read-through, resulting in a truncated protein (D Salvatore et al. 1995). Replacing the Sec-residue in the catalytic center of DIO3 by cysteine dramatically reduced the affinity for T3, while replacement with alanine completely inactivated DIO3 (Kuiper, Klootwijk, and Visser 2003), showing the essential role of the Sec residue for efficient deiodination of T3 (Kuiper, Klootwijk, and Visser 2003; D Salvatore et al. 1995).

As mentioned before, the TH-inactivating DIO3 pathway is highly active during prenatal development, with a tissue distribution much broader than in adults. DIO3 is expressed in most fetal tissues as well as in the placenta to prevent premature T3-induced differentiation and maturation (Gereben et al. 2008; Hernandez et al. 2006). Direct evidence that placental DIO3 activity can in fact limit TH transfer from the maternal blood compartment to the fetal circulation has been obtained in studies carried out in isolated perfused human placental lobules. When T4 was added to the maternal circuit, transfer of T4 to the fetal circuit was very low and the level of rT3 increased progressively in both the fetal and the maternal circuits (Mortimer et
Relatively low tissue levels of TH therefore characterize fetal development, particularly in rodents where fetal thyroid activity only begins several days before birth. Nevertheless, expression of DIO3 and DIO2 appears to be tightly coordinated, so that TH signaling in a given tissue can be controlled according to the developmental stage (reviewed by Galton 2005). The spatiotemporal expression pattern of DIO2 and DIO3 has been shown to be critical for maturation of the mammalian nervous system, the retina and cochlea, and the maturation of the HPT-axis (Ng et al. 2009; Ng et al. 2010; Hernandez et al. 2006). Around birth DIO3 expression is turned off in virtually every tissue, with the exception of skin, the cerebral cortex (Hernandez et al. 2006; Hernandez et al. 2012), hypothalamus (Alkemade et al. 2005) and anterior pituitary (Alkemade et al. 2006). Together with the sharp rise in plasma T3 levels after the first week of postnatal life (Maillet, van Berlo, and Molkentin 2013), TH signaling increases substantially and is involved in driving the fetal-to-adult switch in gene expression.

The involvement of DIO3 in the local reduction of T3 is dependent on the cellular location of the enzyme (Gereben et al. 2008). Hydropathy analysis of human DIO3 revealed a conserved hydrophobic region in the NH$_2$-terminal, indicating DIO3 is an integral membrane protein. Nevertheless, the cellular location of DIO3 remains a matter of debate (Gereben et al. 2008). Observations in monkey hepatocarcinoma cells suggested that DIO3 is located exclusively in the plasma membrane. Additionally it was suggested that the catalytic site was located extracellularly (Baqui et al. 2003). The latter is unlikely since the enzymatic deiodination reaction is a reductive process (Kuiper, Klootwijk, and Visser 2003) and recycling of the oxidized enzyme requires reducing conditions which are not present in the extracellular compartment. Immunohistochemical analysis of DIO3 expression in cardiomyocytes showed DIO3 expression throughout the cytoplasm (Pol et al. 2011), confirming earlier observations made in hemangiomas (S. A. Huang et al. 2005).
Aims and outline of the study

In spite of the tremendous research effort focused on the role of miRNAs in normal cardiac physiology and, particularly, in cardiac disease, very little is known about the effects of T3 on miRNA expression in the heart. This is all the more remarkable given the wide range of effects of T3 on cardiac gene expression and function, and the alterations in cardiac T3 signaling in the failing heart. The principle aim of our studies was therefore to assess the influence of T3 on miRNA expression in the heart in the context of cardiac remodeling. Established mouse models of T3-induced physiological cardiac hypertrophy and pathological remodeling following MI were used to determine cardiac miRNA signatures and to examine possible associations with changing T3 levels in the heart. In addition, we extended previous analyses of cardiac tissue of heart-failure patients to determine the consequences of the observed expression of Dio3.

The following questions were addressed in this study:

Are cardiac miRNAs differentially expressed in response to increasing plasma T3 levels?

Chapter 2 describes the identification of a unique miRNA signature in the mouse left ventricle after short-term T3-treatment of hypothyroid mice. These miRNAs are suggested to suppress pathological signaling typically seen in cardiac hypertrophy.

Are T3-dependent miRNAs involved in post-MI cardiac remodeling?

In chapter 3 we analyzed the miRNA signature of the post-MI mouse left ventricle. Besides confirming the upregulation of DIO3 in the remodeling LV, we found an upregulated cluster of miRNAs located within the DLK1-DIO3 genomic imprinted region. Here, we further elaborate on the possible role of these miRNAs in the proliferation and pluripotency of adult cardiomyocytes.

Is DIO3 activity regulated by miRNAs?

In chapter 4 we show that miRNA-214, which is upregulated in the post-MI heart, targets DIO3 and we identify a feed-back loop that limits DIO3 expression.

Is DIO3 induced in human heart failure?

In chapter 5 we present additional evidence for the re-expression of DIO3 in human end-stage heart failure and its consequence for cardiac TH metabolism.

What are the implications of cardiac TH action for cardiomyocyte fate during physiological hypertrophy and cardiac remodeling following MI?

The results and conclusions of the studies described in this thesis are summarized and discussed in chapter 6. Here, we elaborate on the possibility for a far more general role of TH action in both physiological hypertrophy and cardiac remodeling following MI. In particular we address the role of TH in relation to cardiac regeneration.