IDENTIFICATION OF A ROLE FOR
THE IROQUOIS COMPLEX IN
DROSOPHILA HEART DEVELOPMENT

D I S S E R T A T I O N

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<th>Description</th>
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<tbody>
<tr>
<td>A8</td>
<td>abdominal 8</td>
</tr>
<tr>
<td>Ara</td>
<td>Araucan</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic Helix-Loop-Helix</td>
</tr>
<tr>
<td>Bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<tr>
<td>Brk</td>
<td>Brinker</td>
</tr>
<tr>
<td>Caup</td>
<td>Caupolican</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>double-distilled water</td>
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<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DGRC</td>
<td><em>Drosophila</em> genomics resource center</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxigenin</td>
</tr>
<tr>
<td>DI</td>
<td>Delta</td>
</tr>
<tr>
<td>Dmef2</td>
<td><em>Drosophila</em> myocyte enhancer factor 2</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>Doc</td>
<td>Dorsocross</td>
</tr>
<tr>
<td>Dpp</td>
<td>Decapentaplegic</td>
</tr>
<tr>
<td>DTAF</td>
<td>Dichlorotriazinylamino fluorescein</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
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<tr>
<td>En</td>
<td>Engrailed</td>
</tr>
<tr>
<td>Eve</td>
<td>Even-skipped</td>
</tr>
<tr>
<td>FasIII</td>
<td>Fasciclin III</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
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<tr>
<td>H</td>
<td>Hour</td>
</tr>
<tr>
<td><em>hand</em>-luc</td>
<td><em>hand</em>-luciferase reporter construct</td>
</tr>
<tr>
<td>HD</td>
<td>Homeodomain</td>
</tr>
<tr>
<td>Hh</td>
<td>Hedgehog</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>Iro-C</td>
<td>Iroquois-complex</td>
</tr>
<tr>
<td>Irx</td>
<td>Iroquois homeobox (transcription factor; in vertebrates)</td>
</tr>
<tr>
<td>JAK/Stat</td>
<td>Janus kinase/signal transducers and activators of transcript</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
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Foreword

“The behaviour of the cell in an embryo depends on the extent to which it listens to its mother or its neighbourhood. The size and the nature of the noise, the way in which it is heard and the response are unpredictable and can only be discovered by experimentation.”

1 Introduction

1.1 Heart development in *Drosophila melanogaster*

Understanding how a complex gene network controls cardiogenesis is one of the important questions in developmental biology. Different model organisms are used to identify and characterize signalling pathways and factors that are involved in heart development. The heart of *Drosophila melanogaster*, which is also called dorsal vessel, is a powerful system for studying mechanisms that control cardiogenesis, although the morphology of the fly heart is simpler compared to the multichambered mammalian heart. The *Drosophila* heart is the organ that pumps the hemolymph, a fluid that is analogous to blood, in an open circulatory system. Note that in contrast to vertebrates, the circulating hemolymph supplies the fly body with nutrients, but not primarily with oxygen. In contrast to vertebrate embryos, *Drosophila* embryos develop without a functional heart. The dorsal vessel becomes contractile when the embryos enter the larval stages. It is noteworthy that the molecular network that governs early stages of cardiogenesis is largely conserved between *Drosophila* and vertebrates. Therefore, the identification and characterization of the role of novel components required for heart development in *Drosophila* may be beneficial for a better understanding of mechanisms, controlling early stages of cardiogenesis in vertebrates.

1.1.1 Early steps of heart development: the role of growth factors

The mesodermal germ layer is generated during gastrulation of the blastoderm stage embryo. The mesoderm gives rise to different tissues and organs, such as the heart, visceral musculature, dorsal and ventral body wall muscles (Bate, 1993; Frasch and Nguyen, 1999). The correct formation of the mesoderm and its derivatives depends on the coordinated spatial and temporal expression and interaction of multiple transcription factors and signalling pathways. Many transcription factors and signalling pathways involved in the early steps of cardiogenesis are conserved between flies and vertebrates and are summarized in Table 1. Growth factors that are important for normal mesoderm formation and differentiation of mesodermal cells include Decapentaplegic (Dpp), a
member of the TGF-β family, Wingless (Wg), a member of the Wnt family, Hedgehog (Hh) and Notch (N) (Carmena et al., 2002; Frasch, 1995; Halfon et al., 2000; Jagla et al., 2002; Riechmann et al., 1997; Wu et al., 1995). In addition, the Ras/mitogen-activated protein kinase (MAPK) pathway that can be initiated by signalling through the fibroblast growth factor receptor (FGFR) functions in the generation of distinct subsets of mesodermal cells (Beiman et al., 1996; Bryantsev and Cripps, 2009; Halfon et al., 2000; Liu et al., 2006).

Table 1. Growth factors and transcription factors that control cardiogenesis in *Drosophila* and their functional homologs in vertebrates.

<table>
<thead>
<tr>
<th><em>Drosophila</em></th>
<th>Vertebrates</th>
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<tbody>
<tr>
<td>FGF</td>
<td>FGF</td>
</tr>
<tr>
<td>Dpp</td>
<td>BMP-4</td>
</tr>
<tr>
<td>Wg</td>
<td>Wnt</td>
</tr>
<tr>
<td>Notch</td>
<td>Notch</td>
</tr>
<tr>
<td>Hh</td>
<td>Shh</td>
</tr>
<tr>
<td>Tin</td>
<td>Nkx2-5</td>
</tr>
<tr>
<td>Pnr</td>
<td>GATA4</td>
</tr>
<tr>
<td>Doc1/2/3</td>
<td>Tbx5</td>
</tr>
<tr>
<td>Tup</td>
<td>Isl1</td>
</tr>
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</table>

During *Drosophila* gastrulation, mesodermal cells invaginate and spread laterally. The spreading of the mesodermal layer is controlled by *Drosophila* FGF signalling. Lack of heartless, the *Drosophila* FGF receptor, results in a failure of mesoderm formation and in a complete loss of its derivatives, including the heart (Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1997).

The spreading of the mesoderm is important, because the most lateral cells will migrate dorsally and will receive signals from the ectoderm that specifies mesodermal cell lineages. During early embryogenesis, the dorsal region of the mesoderm receives signals from the ectodermal growth factor Dpp, which is essential for dorsal mesoderm specification (Figure 1 A) (Frasch, 1995). Embryos that are mutant for *dpp* are characterized by a complete loss of cardiac and visceral mesodermal cells (Frasch, 1995). Dpp is secreted from the dorsal ectoderm and induces the expression of the homeobox transcription factor *tinman* (*tin*) in the underlying mesoderm (Lockwood and Bodmer, 2002; Xu et al., 1998; Yin and Frasch, 1998). Consequently, embryos that are mutant for
tin also lack dorsal mesoderm derivatives (Azpiazu and Frasch, 1993; Bodmer, 1993; Zaffran and Frasch, 2002).

As development proceeds, the dorsal mesoderm also receives signals from the growth factor Wg that is secreted from the ectoderm. Wg is expressed in segmentally arranged stripes perpendicular to Dpp (Figure 1 A and Lockwood and Bodmer, 2002) and the Tin-positive dorsal mesodermal cells that receive both Dpp and Wg signals, are determined to become cardiac mesoderm (Park et al., 1996; Wu et al., 1995). Mesodermal cells that do not receive the Wg signal and are under the influence of Hh and Dpp have a visceral mesodermal fate (Azpiazu et al., 1996). Wg function is critical for cardiogenesis, since a wg loss-of-function mutation causes a loss of cardiac progenitors (Wu et al., 1995). Hh is also involved in the induction of cardiac mesoderm, however indirectly, by maintaining wg expression during this process (Park et al., 1996).

During stage 11, the initially continuous expression of tin along the dorsal side of the embryo becomes interrupted segmentally along the anterior-posterior axis (Figure 1 B). Additionally, Tin-positive cells segregate into a dorsal and ventral cluster in each segment. The dorsal cluster contains the cardiac precursor cells whereas the ventral cluster contains Tin-positive cells that are part of the visceral mesoderm (Figure 1 B). Subsequently, the
cardiac precursors align into two continuous rows of heart cells on both sides of the embryo. From stage 13 on, the two rows of heart cells move dorsally towards each other (Figure 1 C), meet at the dorsal midline and form the dorsal vessel by stage 16 (Figure 1 D).

The mature dorsal vessel consists of a defined number of myocardial and pericardial cells. The number of myocardial and pericardial cells is restricted by Notch (N) signalling at early stages of cardiogenesis. The functions of N in Drosophila heart development are: first, to limit the number of cardiac progenitors and second, to regulate lineage decisions in the specification of myocardial cells (Carmena et al., 2002; Han and Bodmer, 2003; Hartenstein et al., 1992; Zaffran and Frasch, 2002). The specification and maintenance of all cardiogenic lineages also require MAPK signalling that is activated by both EGFR and FGFR (Grigorian et al., 2011). The expression of the Notch ligand Delta (Dl) in the cardiogenic clusters is maintained by an activated MAPK pathway and therefore MAPK signalling controls the lineage decision of heart progenitors (Grigorian et al., 2011). The receptor tyrosine kinase (RTK) pathway (including the FGF receptor Heartless) and Ras (G-protein) and MAPK signalling were shown also to regulate eve expression in the mesoderm (Carmena et al., 2002; Halfon et al., 2000).

The Drosophila heart is a simple linear tube that spans from the thoracic segment T2 to abdominal segment A8 (Figure 2 A). The posterior portion of the heart has a wider lumen and is named “heart proper”. The part anterior to the “heart proper” is called “aorta”. A valve separates the “aorta” from the “heart proper” and prevents the backflow of the hemolymph. The heart of Drosophila is composed of two major cell types: the contractile myocardial cells and the non-contractile pericardial cells. Myocardial cells form the inner layer of the heart and pump the hemolymph in a posterior to anterior direction into the body cavity. The hemolymph reenters the heart through three pairs of ostia (inflow tracts), located in the heart proper. Pericardial cells lie adjacent to the myocardial cells and the ultrastructure of pericardial cells suggests a role for these cells in ultrafiltration of the hemolymph and as secretory cells (Mills and King, 1965). The lymph glands, the hematopoetic organ and the endocrine ring glands are located at the anterior part of the fly heart. Additionally, there are seven pairs of alary muscles that attach the heart to the dorsal epidermis (Rugendorff et al., 1994).

Despite of its simple morphology, the dorsal vessel has genetically diverse cells that are arranged segmentally in a stereotypic manner. Therefore it is possible to label different heart cells by using different combinations of molecular markers (Figure 2 C).
For example, there are six myocardial cells present in each hemisegment. All six of them express the *Drosophila* myocyte enhancer factor 2 (Dmef2) and the LIM-homeodomain transcription factor Tailup (Tup) (Bour et al., 1995; Lilly et al., 1995; Mann et al., 2009) (Figure 2 B, C), whereas only four of them are characterized by the expression of Tinman (Tin) (Azpiazu and Frasch, 1993; Bodmer, 1993) (Figure 2 A, C). The two posterior Tin-expressing myocardial cells also express Ladybird early and Ladybird late homeobox genes (Lb) (Jagla et al., 1997). The remaining two myocardial cells, that are Tin-negative, express the orphan nuclear receptor Seven-up (Svp) and the T-box factor Dorsocross (Doc) (Molina and Cripps, 2001; Reim and Frasch, 2005). The Zinc-finger homeodomain-1 protein (Zfh-1) demarcates all pericardial cells at stage 16 (Lai et al., 1991) (Figure 2 B, C). A subset of pericardial cells and the lymph gland cells are characterized by expression of the pair-rule gene Odd-skipped (Odd) and the transcription factor Tup (Mann et al., 2009; Ward and Skeath, 2000).

![Figure 2. The structure of the *Drosophila* heart and some molecular markers that can be used to distinguish different heart cells.](image)

(A) A schematic illustration of the dorsal vessel is shown. Two morphologically distinct regions, the aorta and the heart proper, are indicated. Myocardial cells (MC) form the contractile tube and are surrounded by pericardial cells (PC). The flow of hemolymph is shown by arrows. Of note, there are more pericardial cells than shown in this illustration. (B) A stage 16 wildtype embryo where all MCs are visualized by the expression of Dmef2 and PCs are visualized by the expression of Zfh-1. Zfh-1 expression is also detected in the lymph glands (lg). Anterior is to the left. (C) A summary of heart cells present in one hemisegment of the dorsal vessel at late embryonic stages, where the MCs and PCs are characterized by specific combinations of transcription factors presented in the cartoon (Gajewski et al., 2000; Jagla et al., 1997; Jagla et al., 2002; Lilly et al., 1995; Mann et al., 2009; Su et al., 1999; Ward and Skeath, 2000).
Odd and Tup mark four pericardial cells in each hemisegment and among these Odd cells, two out of the four also express Svp. The other six pericardial cells that are negative for Odd, express Tin alone or in combination with either Lb or Eve. Yet another transcription factor Hand starts to be detectable in the developing heart at stage 12 and by stage 15/16 it is expressed in all myocardial and pericardial cells (Han and Olson, 2005).

The diversity of factors allows for the characterization of heart phenotypes in embryos that are mutant for the gene of interest.

1.1.2 The role of cardiac transcription factors during cardiogenesis

The orchestrated action of signalling pathways activated by the aforementioned growth factors contributes to the establishment of a complex cardiac transcriptional network. The following transcription factors are the core set of factors that are crucial for normal heart development.

The Nk-homeobox gene tinman

Tinman (Tin) exhibits a dynamic expression pattern during cardiogenesis, which demonstrates a role for this factor in different steps of heart development. Tin is present throughout the mesoderm at early embryonic stages and plays a role in the patterning of the dorsal mesoderm and specification of its derivatives (Azpiazu and Frasch, 1993; Bodmer, 1993; Yin and Frasch, 1998). The expression of tin is regulated by different factors during embryogenesis and can be divided into three phases. The early pan-mesodermal tin expression is directly regulated by the mesoderm-inducing transcription factor Twist (Yin et al., 1997) and by autoregulation (Xu et al., 1998). In the second phase, stage 10, tin expression becomes restricted to the dorsal mesoderm by Dpp signalling (Frasch, 1995; Xu et al., 1998; Yin and Frasch, 1998). Embryos that lack tin during these two phases are devoid of the tissues that arise from the dorsal mesoderm (Zaffran et al., 2002). When the presumptive cardiac tissue has formed, the initial broad expression of Tin becomes fragmented segmentally and it is detected in two distinct cell clusters: in the dorsally located heart precursors and in more lateral cells that correspond to visceral mesoderm (Lockwood and Bodmer, 2002). Slightly later, tin expression is only present in the heart precursors. By stage 15/16, which marks the third phase of tin expression, its expression is maintained in most of the myocardial cells and in a subset of pericardial cells.
(Figure 2 C). At this step of cardiogenesis, the normal expression of tin in the developing myocardial cells is required for the proper diversification of distinct heart cell types as well as for their differentiation (Zaffran et al., 2006).

Despite the crucial role of Tin during early cardiogenesis, additional factors are required for normal heart development. The proper formation of cardiac progenitors requires the coordinated action of the key player tin and the three T-box related Dorsocross1-3 (Doc1-3) genes, the LIM homeobox transcription factor tailup (tup) and the GATA transcription factor pannier (pnr) (Alvarez et al., 2003; Gajewski et al., 1999; Klinedinst and Bodmer, 2003; Mann et al., 2009; Reim and Frasch, 2005; Tao et al., 2007).

The T-box related genes Dorsocross 1-3

The three Doc genes (Doc 1-3) share similarities in their T-box sequences and have a similar expression pattern in a number of tissues including the dorsal mesoderm (Reim et al., 2003). At stage 10 they are expressed in the dorsal mesoderm in segmentally arranged cell patches. This early expression of Doc 1-3 has been demonstrated to be dependent on the Dpp and Wg signalling pathways (Reim and Frasch, 2005). At stage 11/12, Doc is co-expressed with Tin in the majority of heart cells. From mid-embryogenesis onward, Doc expression is restricted to one pair of myocardial cells per hemisegment. These two cells co-express Svp but are Tin-negative and develop into an inflow tract in the heart proper (Gajewski et al., 2000; Lo and Frasch, 2001) (see Figure 2 A, C). Reim and Frasch (2005) demonstrated that Doc is required for the specification of myocardial and pericardial cells (except for eve-expressing cells), since the embryos that lack all three Doc genes are characterized by a severe cardiac phenotype.

The GATA transcription factor pnr

The GATA factor pnr is detected in the dorsal ectoderm and in the dorsal mesoderm at stage 10 (Gajewski et al., 1999) and its function in both ecto- and mesoderm have been shown to be important for the development of the dorsal vessel (Klinedinst and Bodmer, 2003). Pnr and the mouse GATA4 protein share similarities in their two zinc finger sequences and moreover, mouse GATA4 can functionally replace pnr in cardiogenesis, when it is expressed in the mesoderm of Drosophila embryos (Gajewski et al., 1999). At stage 10/11, Dpp and Tin regulate expression of pnr in the dorsal mesoderm, where pnr function is needed for heart cell specification (Klinedinst and Bodmer, 2003). In fact, pnr is a direct transcriptional target of Tinman (Gajewski et al., 2001). However, pnr
may also mediate, at least in part, the cardiogenic Dpp signal in the mesoderm (Klinedinst and Bodmer, 2003). Embryos mutant for pnr are characterized by severe defects in cardiac cell specification resulting in reduced numbers of myocardial and pericardial cells. (Alvarez et al., 2003; Gajewski et al., 1999; Klinedinst and Bodmer, 2003).

**The LIM homeodomain transcription factor tailup (tup)**

The *Drosophila* transcription factor *tup*, ortholog of vertebrate *Islet1*, is another component of the *Drosophila* early cardiac transcriptional network (Mann et al., 2009; Tao et al., 2007). The expression of *tup* is detected at early steps of heart development (stage 11) in the cardiac mesoderm and the correct specification of heart cells has been demonstrated to be dependent on *tup* expression (Mann et al., 2009). At stage 11, *tup* is also expressed in the ectoderm and similar to *pnr*, *tup* expression in both, in the ecto- and in the mesoderm, is required for the proper development of the *Drosophila* heart (Klinedinst and Bodmer, 2003; Mann et al., 2009). After the heart cells have been specified, *tup* is expressed in all myocardial cells and in the Odd-expressing pericardial cells, as well as in the Odd-positive lymph gland cells (Mann et al., 2009; Tao et al., 2007). At stage 15/16, *tup* mutants exhibit a reduction of myocardial and pericardial cells, demonstrating the importance of *tup* function for the formation of the dorsal vessel (Mann et al., 2009).

**1.1.3 Genetics of cardiac cell specification**

*Drosophila* owns a complex gene network that governs cardiogenesis. The above mentioned signalling pathways and transcription factors act in combination and are interdependent. Dpp and Wg signals induce the expression of important cardiac transcription factors *tin*, *pnr*, *Doc* as well as *tup* and thereby specify the cardiac mesoderm (Frasch, 1995; Klinedinst and Bodmer, 2003; Mann et al., 2009; Park et al., 1996; Reim and Frasch, 2005; Wu et al., 1995; Yin and Frasch, 1998). *Doc* and *tin* are required to induce the mesodermal expression of *pnr* (Gajewski et al., 2001; Reim and Frasch, 2005). The maintenance of *tin* and *Doc* in cardiac precursors depends on the presence of *pnr* (Klinedinst and Bodmer, 2003; Reim and Frasch, 2005). Subsequently, *tin* and *pnr* cooperate to activate expression of their direct target *Dmef2* (MADS box transcription factor) which is involved in heart cell differentiation (Gajewski et al., 1997; Gajewski et
al., 2001). Mann et al. (2009) demonstrated that the expression of Doc, pnr and tin depends on tup at stage 11 and conversely the expression of tup requires the presence of Doc, pnr and tin transcription factors. The bHLH factor hand is a direct target of crucial cardiogenic factors tin, pnr and tup (Han and Olson, 2005; Tao et al., 2007). Whereas lack of the bHLH factor hand has a minor effect on the embryonic heart development, hand has been shown to affect the adult Drosophila heart morphology and function which suggests a role for hand in the cardiac cell differentiation process (Han et al., 2006; Lo et al., 2007; Reim and Frasch, 2010).

Thus, the regulated spatial and temporal expression of transcription factors and their target genes within the heart forming region ensures the correct development of the dorsal vessel. Once the Drosophila heart tube is formed it starts to contract and pumps hemolymph through the organism in an open circulatory system.

1.2 Introduction to the Iroquois-complex of homeodomain transcription factors

Iroquois transcription factors (Iro/Irx) are found in almost all Metazoan species and belong to the TALE (three amino acid loop extension) superclass of homeobox genes, harbouring 63 amino acids in their highly conserved homeodomain (Bürglin, 1997). Six Iroquois genes (Irxi-6) exist in most vertebrates. In mammals, Irx family members are grouped in two gene clusters IrxA and IrxB, each cluster, containing three genes, located on mouse chromosomes 13 and 8 and on human chromosomes 5 and 16 (Cavodeassi et al., 2001; Gomez-Skarmeta and Modolell, 2002; Peters et al., 2000). The vertebrate Irx family members function in diverse developmental processes, such as specification and patterning of different tissues and organs (Bao et al., 1999; Cavodeassi et al., 2001; Cheng et al., 2006; Itoh et al., 2002; Joseph, 2004; Kim et al., 2012; Rodriguez-Seguel et al., 2009). The Drosophila Iroquois complex (Iro-C) consists of the three homeobox transcription factors araucan (ara), caupolican (caup) and mirror (mirr) (Gomez-Skarmeta et al., 1996; McNeill et al., 1997). Members of the Iro-C are clustered together and located on the Drosophila third chromosome. Iro-C proteins share highly conserved domains: the homeodomain (TALE) and the EGF-like motif (Cavodeassi et al., 2001; Gomez-Skarmeta et al., 1996; McNeill et al., 1997). The EGF-like motif represents a potential protein-
protein interaction domain which is similar to those of Notch proteins (Gomez-Skarmeta et al., 1996; McNeill et al., 1997). Another feature of Ara and Caup is the presence of putative phosphorylation sites for MAPK (Gomez-Skarmeta et al., 1996). In fact, MAPK modulates the transcriptional activity of Caup and of chicken Irx2 (Carrasco-Rando et al., 2011; Matsumoto et al., 2004). The comparison of protein sequences of Ara, Caup and Mirr showed that Ara and Caup are more similar one to another and share 41% overall identity, while Mirr has only 26% sequence identity with Ara and 29% with Caup. However, all three Iro-C proteins show over 92% identity in their homeodomain region. In addition, ara and caup have a similar expression pattern (Carrasco-Rando et al., 2011; Gomez-Skarmeta et al., 1996), share cis-regulatory regions and act redundantly in most of the cases (Cavodeassi et al., 2001; Ikmi et al., 2008). The third member of Iro-C, mirr, is expressed in tissues different from where ara and caup are expressed (Ikmi et al., 2008; McNeill et al., 1997).

Based on previous studies, the Drosophila Iro-C members are important for patterning of the notum, for the specification of dorsal head structures and for the dorso-ventral subdivision of the eye and ovary (Calleja et al., 2000; Calleja et al., 2002; Cavodeassi et al., 1999; Cavodeassi et al., 2000; Ikmi et al., 2008; Jordan et al., 2000; McNeill et al., 1997; Pichaud and Casares, 2000; Yang et al., 1999). Another function of Iro-C during development is their requirement for the specification and differentiation of diverse structures like wing veins, the fly sensory organs and the lateral transverse muscles (Carrasco-Rando et al., 2011; Gomez-Skarmeta et al., 1996). The Drosophila Iro-C genes cooperate with tup to specify the dorsal mesothorax in the wing imaginal disc (de Navascues and Modolell, 2007). Additionally, pnr has been demonstrated to regulate the expression Iro-C in the prospective notum region of the wing disc (Letizia et al., 2007). Thus, the Drosophila Iro-C has been shown to interact with factors that are important for the development of the early cardiogenic mesoderm. This suggests that tup and pnr may interact with Iro-C in heart development.

It is known that vertebrate Irx transcription factors are expressed in the developing heart (Christoffels et al., 2000; Kim et al., 2012). Irx1-6 are expressed in an overlapping manner as well as in diverse structures of the cardiac tissue (Christoffels et al., 2000; Kim et al., 2012). This suggests the possible contribution of Irx to the development of specific structures of the heart. However, lack of either Irx factor in mice (single knockout) does not result in a dramatic cardiac phenotype suggesting a redundant role for these genes (Kim et al., 2012). Nevertheless, Irx3 is required for correct electrophysiological properties
of the mouse heart (Zhang et al., 2011) and mice lacking *Irx4* develop an adult cardiomyopathy (Bruneau et al., 2001). Whereas single *Irx3* or *Irx5* knockout mice are characterized by mild cardiac defects (Costantini et al., 2005; Zhang et al., 2011) the lack of both, *Irx 3* and *Irx 5*, results in severe cardiac defects and prenatal death demonstrating functional redundancy of *Irx3* and *Irx5* and the requirement of these factors for cardiogenesis (Gaborit et al., 2012). The expression pattern and function of vertebrate *Irxl-6* in the developing heart is evident (Kim et al., 2012), however little is known about the mechanisms that control *Irx* expression in the heart and about the targets of *Irx*.

Thus, investigation of the role of *Drosophila Iro-C* in heart development may bring new insights into mechanisms and the function of *Irx* factors in the vertebrate heart.

### 1.3 Aims of the study

The focus of this work was to investigate the possible role of *Iro-C* in *Drosophila* heart development, as it has not been analyzed yet. To explore the function of the members of *Iro-C* in dorsal vessel development the following topics were addressed:

1. Detailed analyses of the embryonic expression pattern of Ara, Caup and Mirr during cardiogenesis.
2. To determine the impact of the lack of *Iro-C* on *Drosophila* heart development.
3. To analyze the interdependency of *Iro-C* and core cardiogenic factors in order to determine whether *Iro-C* can be integrated into the early cardiac transcriptional network.
4. To identify possible direct targets of *Iro-C*.
## 2 Materials and Methods

### 2.1 Materials

#### 2.1.1 Reagents list

<table>
<thead>
<tr>
<th>Name</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>Fluka</td>
</tr>
<tr>
<td>Agarose</td>
<td>Lonza</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Roth</td>
</tr>
<tr>
<td>Bacto-Agar (embryo collection plates)</td>
<td>Fluka</td>
</tr>
<tr>
<td>Bleach</td>
<td>DanKlorix, Colgate-Palmolive</td>
</tr>
<tr>
<td>Boric Acid</td>
<td>Sigma</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Ambion</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Ambion</td>
</tr>
<tr>
<td>Denhardts (50x)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Diethylpyrocarbonat (DEPC)</td>
<td>AppliChem, Sigma</td>
</tr>
<tr>
<td>Dimethylsulfoxide (DMSO)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Dithiothreitol (DTT)</td>
<td>Sigma</td>
</tr>
<tr>
<td>DNA-gene ruler</td>
<td>Fermentas</td>
</tr>
<tr>
<td>DNase I</td>
<td>Roche</td>
</tr>
<tr>
<td>EDTA</td>
<td>Sigma</td>
</tr>
<tr>
<td>EGTA</td>
<td>Sigma</td>
</tr>
<tr>
<td>Ethanol (not absolute)</td>
<td>Pharmacy</td>
</tr>
<tr>
<td>Ethanol absolute</td>
<td>Riedel-de Haën</td>
</tr>
<tr>
<td>Ethidium bromide (10mg/ml)</td>
<td>Roth</td>
</tr>
<tr>
<td>Fetal Bovine Serum (FBS)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Formaldehyde 20%</td>
<td>Merck</td>
</tr>
<tr>
<td>Formamide</td>
<td>Applichem</td>
</tr>
<tr>
<td>Glutathione Sepharose-4B</td>
<td>Amersham</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Roth</td>
</tr>
<tr>
<td>Glycine</td>
<td>Sigma</td>
</tr>
<tr>
<td>HEPES</td>
<td>Roth</td>
</tr>
<tr>
<td>Heptane</td>
<td>Merck</td>
</tr>
<tr>
<td>Hydrochloric acid (HCl)</td>
<td>Merck</td>
</tr>
<tr>
<td>Hydrogen peroxide 30%</td>
<td>Merck</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Merck</td>
</tr>
<tr>
<td>LB Agar</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>LB Broth Base</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Loading dye (6x)</td>
<td>Fermentas</td>
</tr>
<tr>
<td>Magnesium chloride (MgCl₂)</td>
<td>Merck</td>
</tr>
<tr>
<td>Methanol</td>
<td>Merck, Fluka</td>
</tr>
<tr>
<td>NP-40</td>
<td>USB</td>
</tr>
<tr>
<td>Paraformaldehyde (PFA)</td>
<td>Roth</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>Merck</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>Roch</td>
</tr>
<tr>
<td>Potassium chloride (KCl)</td>
<td>Merck</td>
</tr>
<tr>
<td>Poly dIdC</td>
<td>Sigma</td>
</tr>
</tbody>
</table>
# Materials and Methods

**PD098059**
*Sigma*

**RNAOut**
*Invitrogen*

**RNase A**
*Roch*

**Schneider's *Drosophila* Medium**
*Invitrogen*

**Sodium acetate Trihydrate**
*Merck*

**Sodium chloride**
*Applichem*

**Sodium dihydrogen phosphate Monohydrate (NaH$_2$PO$_4$ x H$_2$O)**
*Fluka*

**Sodium hydroxide**
*Merck*

**Sodium phosphate dibasic Dihydrate (Na$_2$HPO$_4$ x 2H$_2$O)**
*Fluka*

**Tris-hydroxymethylaminomethane (Tris)**
*Sigma*

**Triton X-100**
*Merck*

**Tween 20 (Polyoxyethylensorbitanmonolaureate)**
*Merck*

**X-Gal**
*Promega*

**Xylene**
*Merck*

## 2.1.2 Cells

<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> cell strains: One Shot TOP 10; BL21</td>
<td>Invitrogen; Stratagene</td>
</tr>
<tr>
<td><em>Drosophila</em> Schneider 2 (S2) cells</td>
<td><em>Drosophila</em> Genomics Resource Center (DGRC)</td>
</tr>
</tbody>
</table>

## 2.1.3 Kits

<table>
<thead>
<tr>
<th>Name</th>
<th>Producer</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIG RNA Labeling Mix 10x</td>
<td><em>Roche</em></td>
</tr>
<tr>
<td>DNA polymerases: Pfu Ultra II Fusion HS DNA-polimerase; Phire Hot Start II PCR Master Mix; GoTaq PCR kit</td>
<td><em>Stratagene; Finnzymes; Promega</em></td>
</tr>
<tr>
<td>Dual-Luciferase Reporter Assay System</td>
<td><em>Promega</em></td>
</tr>
<tr>
<td>DYEnamic ET Terminator Cycle Sequencing Kit</td>
<td><em>Amersham</em></td>
</tr>
<tr>
<td>FectoFly</td>
<td><em>Polyplus</em></td>
</tr>
<tr>
<td>GeneJET PCR Purification Kit</td>
<td><em>Fermentas</em></td>
</tr>
<tr>
<td>GeneJET Gel Extraction Kit</td>
<td><em>Fermentas</em></td>
</tr>
<tr>
<td>Ligate-IT Rapid Ligation Kit</td>
<td><em>USB</em></td>
</tr>
<tr>
<td>QIAfilter Midi plasmid purification kit</td>
<td><em>Qiagen</em></td>
</tr>
<tr>
<td>Restriction enzymes and buffers</td>
<td><em>New England BioLabs (NEB)</em></td>
</tr>
<tr>
<td>RNA magic</td>
<td><em>Bio-Budget</em></td>
</tr>
<tr>
<td>RNA polymerase T3</td>
<td><em>Roche</em></td>
</tr>
<tr>
<td>Superscript III First-Strand Synthesis System for RT-PCR</td>
<td><em>Invitrogen</em></td>
</tr>
<tr>
<td>T4 Polynucleotide Kinase kit</td>
<td><em>Fermentas</em></td>
</tr>
<tr>
<td>TSA Biotin System NEL700A</td>
<td><em>Perkin Elmer</em></td>
</tr>
<tr>
<td>Wizard Plus SV Minipreps DNA Purification System</td>
<td><em>Promega</em></td>
</tr>
</tbody>
</table>
2.1.4 Equipment

<table>
<thead>
<tr>
<th>Apparatus</th>
<th>Model</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Sequencer</td>
<td>ABI Prism 377</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Biosafety cabinet, class II</td>
<td>Type A; B3 (bacteria; flies)</td>
<td>NuAire</td>
</tr>
<tr>
<td>Block heater</td>
<td>HBT 130</td>
<td>HLC</td>
</tr>
<tr>
<td>CO₂ tanks</td>
<td></td>
<td>MTI Industriegase AG</td>
</tr>
<tr>
<td>Centrifuges</td>
<td>5415 R; 5424</td>
<td>Eppendorf</td>
</tr>
<tr>
<td></td>
<td>Megafuge 1.0R</td>
<td>Heraeus</td>
</tr>
<tr>
<td></td>
<td>Biofuge primo R</td>
<td>Heraeus</td>
</tr>
<tr>
<td></td>
<td>Sorvall RC-5B</td>
<td>DuPont Instruments</td>
</tr>
<tr>
<td>Confocal laser microscope</td>
<td>TCS SP5 II</td>
<td>Leica</td>
</tr>
<tr>
<td>Digital Balance</td>
<td>EW4200</td>
<td>Kern</td>
</tr>
<tr>
<td>Electrophoresis chamber</td>
<td>Agarose gels</td>
<td>Workshop, Uni Göttingen</td>
</tr>
<tr>
<td>Freezer (-20 °C)</td>
<td></td>
<td>Liebherr</td>
</tr>
<tr>
<td>Freezer (-80 °C)</td>
<td>VX490E</td>
<td>Jovan</td>
</tr>
<tr>
<td>Ice-machine</td>
<td>AF-10</td>
<td>Scotsman</td>
</tr>
<tr>
<td>Imaging system</td>
<td>Gel documentation</td>
<td>LTF Labortechnik</td>
</tr>
<tr>
<td></td>
<td>BAS100 (phosphoimage)</td>
<td>Fujifilm</td>
</tr>
<tr>
<td>Incubators</td>
<td>Kelvitron (bacteria)</td>
<td>Heraeus</td>
</tr>
<tr>
<td></td>
<td>RD3; APT.Line KB (fly)</td>
<td>Binder</td>
</tr>
<tr>
<td></td>
<td>WB22K (cell culture)</td>
<td>Mytron</td>
</tr>
<tr>
<td>Luminometer</td>
<td>EG&amp;G BERTHOLD</td>
<td>Lumat LB 9507</td>
</tr>
<tr>
<td>Magnetic stirrers</td>
<td>RCT (hot-plate)</td>
<td>IKAMAG</td>
</tr>
<tr>
<td></td>
<td>MR Hei-Standard</td>
<td>Heidolph</td>
</tr>
<tr>
<td>Microscopes</td>
<td>Binocular</td>
<td>H. Saur</td>
</tr>
<tr>
<td></td>
<td>BX60</td>
<td>Olympus</td>
</tr>
<tr>
<td></td>
<td>BZ 8000-K</td>
<td>Keyence</td>
</tr>
<tr>
<td></td>
<td>SZX10</td>
<td>Olympus</td>
</tr>
<tr>
<td>Microwave oven</td>
<td>Micromat</td>
<td>AEG</td>
</tr>
<tr>
<td>NanoPhotometer</td>
<td>Perl</td>
<td>Implen</td>
</tr>
<tr>
<td>PCR machines</td>
<td>T3</td>
<td>Biometra</td>
</tr>
<tr>
<td></td>
<td>T Gradient</td>
<td>Biometra</td>
</tr>
<tr>
<td>pH-meter</td>
<td>pH 211</td>
<td>Hanna Instruments</td>
</tr>
<tr>
<td>Photometer</td>
<td>Genesys 10 Bio</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Pipette controller</td>
<td>Pipetus</td>
<td>Hirschmann Laborgeräte</td>
</tr>
<tr>
<td>Pipettes 1000 µl, 200 µl, 20 µl, 10 µl</td>
<td>Labmate Optima</td>
<td>Abimed</td>
</tr>
<tr>
<td>Refrigerator</td>
<td></td>
<td>Liebherr</td>
</tr>
<tr>
<td>Video monitor</td>
<td>PM-K9 (gel documentation)</td>
<td>IKEAGAM</td>
</tr>
<tr>
<td>Video printer</td>
<td>P 91 (gel documentation)</td>
<td>Mitsubishi</td>
</tr>
<tr>
<td>Vortexer</td>
<td>Vortex-Genie2</td>
<td>Scientific Industries</td>
</tr>
<tr>
<td>Water bath</td>
<td>P14</td>
<td>Kaake</td>
</tr>
<tr>
<td>Water purification system</td>
<td></td>
<td>Elga</td>
</tr>
<tr>
<td>Waving platform shaker</td>
<td>Polymax 1040</td>
<td>Heidolph</td>
</tr>
</tbody>
</table>
### 2.1.5 Fly husbandry

<table>
<thead>
<tr>
<th>Product</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>AeraSeal Films (for fly beds)</td>
<td>Genesee Scientific</td>
</tr>
<tr>
<td>Agar-Agar (fly food)</td>
<td>Roth</td>
</tr>
<tr>
<td>Cornflour</td>
<td>Gut &amp; Gerne, BZ Bio-Zentrale</td>
</tr>
<tr>
<td>Cottonballs for small fly vials</td>
<td>Genesee Scientific</td>
</tr>
<tr>
<td>Fly food cooking system</td>
<td>University of Ulm workshop</td>
</tr>
<tr>
<td>Fly-beds</td>
<td>Genesee Scientific</td>
</tr>
<tr>
<td>Gauze</td>
<td>Cheesecloth, Grade 50, Genesee Scientific</td>
</tr>
<tr>
<td>Large fly bottles (L;B 57 mm, H 103 mm, top Ø 42,5 mm)</td>
<td>Square Bottom Bottles, Genesee Scientific</td>
</tr>
<tr>
<td>Nipagin (methyl-4hydroxybenzoate)</td>
<td>Merck</td>
</tr>
<tr>
<td>Oil</td>
<td>Gut &amp; Günstig, Enco (EDEKA)</td>
</tr>
<tr>
<td>Paperlid for fly bottles</td>
<td>Genesee Scientific</td>
</tr>
<tr>
<td>Small fly vials (H 95 mm x 25 Ø mm)</td>
<td>Drosophila Vials, Narrow (PS), Genesee Scientific</td>
</tr>
<tr>
<td>Sugar</td>
<td>Südzucker</td>
</tr>
<tr>
<td>Yeast (fresh or dry)</td>
<td>Dr. Oetker</td>
</tr>
</tbody>
</table>

### 2.1.6 Miscellaneous

<table>
<thead>
<tr>
<th>Miscellaneous</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aluminum foil</td>
<td>Toppits</td>
</tr>
<tr>
<td>Autoclave tapes</td>
<td>Stericlin, Medical Packaging</td>
</tr>
<tr>
<td>Coverslips</td>
<td>Menzel-GLäser</td>
</tr>
<tr>
<td>D-Tube Dialyzer Midi</td>
<td>Novagen</td>
</tr>
<tr>
<td>Glass materials: Bottles, glass pipettes, beakers, Pasteur pipettes, etc.</td>
<td>Brand, Schott, VWR International, Roth</td>
</tr>
<tr>
<td>Gloves (normal, Nitril)</td>
<td>Ansell</td>
</tr>
<tr>
<td>Graduated cylinders</td>
<td>Azlon, VWR International</td>
</tr>
<tr>
<td>Latex pipette bulbs</td>
<td>Azlon, VWR International</td>
</tr>
<tr>
<td>Microcentrifuge tube racks</td>
<td>Brand</td>
</tr>
<tr>
<td>MicroSpin G-50</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>Paintbrush</td>
<td>Schulmalpinsel Nr.1, Ponyhaar, elcopinsel</td>
</tr>
<tr>
<td>Parafilm</td>
<td>American National Can</td>
</tr>
<tr>
<td>Photo processing program</td>
<td>Adobe Photoshop CS3</td>
</tr>
<tr>
<td>Plasticware: Pipette tips, pipettes, microcentrifuge tubes, petri-dishes, Falcon tubes, etc.</td>
<td>Eppendorf, Brand, Greiner, Sarstedt, Corning, Biozym, VWR International</td>
</tr>
<tr>
<td>PVC-funnels</td>
<td>Azlon, VWR International</td>
</tr>
<tr>
<td>Slides</td>
<td>VWR International</td>
</tr>
<tr>
<td>Software for statistic analyses</td>
<td>GraphPad Prism</td>
</tr>
<tr>
<td>Wash bottles</td>
<td>Azlon, VWR International</td>
</tr>
</tbody>
</table>
2.1.7 List of plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Restriction enzyme for linearizing</th>
<th>RNA Polymerase used for RNA synthesis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dpp</td>
<td>BamHI</td>
<td>T3</td>
<td>(Padgett et al., 1987)</td>
</tr>
<tr>
<td>Pnr</td>
<td>HindIII</td>
<td>T3</td>
<td>(Ramain et al., 1993)</td>
</tr>
<tr>
<td>ara</td>
<td>BglII</td>
<td>T3</td>
<td># 9547 DGRC. The additional nucleotide in the ara open reading frame (ORF) was corrected by PCR (this work).</td>
</tr>
<tr>
<td>caup</td>
<td>HindIII</td>
<td>T3</td>
<td>The caup ORF was subcloned into the pBlueScript II KS (+) vector. C. Donow (this work)</td>
</tr>
<tr>
<td>Mirr</td>
<td>NotI</td>
<td>T3</td>
<td>this work</td>
</tr>
</tbody>
</table>

Other plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ara orf in pAc5.1-V5/HisC</td>
<td>The ara ORF was subcloned into pAc5.1-V5/HisC vector (this work)</td>
</tr>
<tr>
<td>caup orf in pAc5.1-V5/HisC</td>
<td>The caup ORF was subcloned into the pAc5.1-V5/HisC vector from the plasmid RE64213 (DGRC) (this work)</td>
</tr>
<tr>
<td>caup HD in pGEX-4T-1</td>
<td>this work</td>
</tr>
<tr>
<td>mirr orf in pAc5.1-V5/HisC</td>
<td>this work</td>
</tr>
<tr>
<td>Hand intron C in pGL4.26</td>
<td>K. Werner</td>
</tr>
<tr>
<td>Renilla</td>
<td>(Matsumoto et al., 2007)</td>
</tr>
</tbody>
</table>

2.1.8 List of antibodies

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Animal</th>
<th>Dilution</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ara/Caup</td>
<td>Rat</td>
<td>1:200</td>
<td>From S. Campuzano (Diez del Corral et al., 1999; Letizia et al., 2007)</td>
</tr>
<tr>
<td>Dmef2</td>
<td>Rabbit</td>
<td>1:2000</td>
<td>From E.N. Olson (Lilly et al., 1995)</td>
</tr>
<tr>
<td>Doc2</td>
<td>Rabbit</td>
<td>1:2000</td>
<td>From M. Frasch (Reim et al., 2003)</td>
</tr>
<tr>
<td>En</td>
<td>Mouse</td>
<td>1:20</td>
<td>Developmental Studies Hybridoma Bank (DSHB)</td>
</tr>
<tr>
<td>Eve (2B8)</td>
<td>Mouse</td>
<td>1:20</td>
<td>DSBH</td>
</tr>
<tr>
<td>Eve</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>From M. Frasch (Frasch et al., 1987)</td>
</tr>
<tr>
<td>FasIII</td>
<td>Mouse</td>
<td>1:2</td>
<td>DSBH</td>
</tr>
<tr>
<td>Isl-1 (Tup)</td>
<td>Mouse</td>
<td>1:20</td>
<td>DSBH</td>
</tr>
<tr>
<td>Mirr</td>
<td>Rabbit</td>
<td>1:500</td>
<td>From H. McNeill (Yang et al., 1999)</td>
</tr>
<tr>
<td>Odd</td>
<td>Rabbit</td>
<td>1:100</td>
<td>From J. B. Skeath (Ward and Skeath, 2000)</td>
</tr>
<tr>
<td>Tin</td>
<td>Rabbit</td>
<td>1:500</td>
<td>From R. Bodmer and M. Frasch (Venkatesh et al., 2000; Yin et al., 1997)</td>
</tr>
<tr>
<td>B-3 Tubulin</td>
<td>Rabbit</td>
<td>1:5000</td>
<td>From R. Renkawitz-Pohl (Kremser et al., 1999)</td>
</tr>
</tbody>
</table>
Materials and Methods

Wg Mouse 1:1 DSHB
Zfh-1 Rabbit 1:5000 (Broihier et al., 1998)
Prc Mouse 1:1 DSHB

<table>
<thead>
<tr>
<th>Secondary Antibody</th>
<th>Coupled with</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat-anti-mouse</td>
<td>Biotin</td>
<td>1:200</td>
<td>Dianova</td>
</tr>
<tr>
<td>Donkey-anti-mouse</td>
<td>Cy3</td>
<td>1:200</td>
<td>Dianova</td>
</tr>
<tr>
<td>Goat-anti-rabbit</td>
<td>Biotin</td>
<td>1:200</td>
<td>Dianova</td>
</tr>
<tr>
<td>Donkey-anti-rabbit</td>
<td>Cy3 or DyLight594</td>
<td>1:200</td>
<td>Dianova</td>
</tr>
<tr>
<td>Sheep-anti-DIG</td>
<td>-</td>
<td>1:200</td>
<td>Roche</td>
</tr>
<tr>
<td>Donkey-anti-sheep</td>
<td>Biotin</td>
<td>1:200</td>
<td>Dianova</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA-DTAF</td>
<td>1:200</td>
<td>Dianova</td>
</tr>
</tbody>
</table>
Streptavidin coupled to 5-(4,6-Dichlorotriazinyl) Aminofluorescin |

2.1.9 List of primers

Unmodified primers for EMSA

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’ - 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caup_BS_hand_1</td>
<td>TTG GTT TGT TAT TGA AAG CAT</td>
</tr>
<tr>
<td>Caup_BS_hand_2</td>
<td>ATG CTT TCA ATA ACA AAC CAA</td>
</tr>
</tbody>
</table>

Unmodified primers for sequencing

F ara_seq_0    | CTG TTA AAC CCC CGA TAA GCA CAT   |
F ara_seq_1    | AGT CCC AAT GCG CTT TCG CA        |
F ara_seq_2    | AAT CCG TAC GGA CTT AAG GAT       |
F ara_seq_3    | AGA TGA CCA TGA TGA CGC CTT       |
F ara_seq_4    | AAA AAC CAG CTA GGC CGG GA        |
F ara_seq_5    | CAG CAA CTG CAG CAG CAA TCT       |
F caup_seq_1   | CTG ACT TTA AAA AAT TTC GAT ACA   |
F caup_seq_2   | CCA GCA AAC AAC AAA AGC GCC       |
F caup_seq_3   | AGT CCA GGT GCT GTT TCC CA        |
F caup_seq_4   | ATA CTA TTC CTA CGA TCC CAC       |
F caup_4a      | GCA ATC AAC TTA TCA AAT CCG A     |
F caup_seq_5   | AAC AGC AGC AGC AGC AGC A         |
F caup_5a      | GAG ACT GTG GCA TCC CAG TT        |
F caup_seq_6   | CTG AGT ATG ATG AGC AGC TAC       |
F mirr_seq_2   | AGG ACA CAG TGG CAT GGT GAC       |
F mirr_seq_3   | GGA GCAT CGC CCT GGC CA           |
F mirr_seq_4   | GAA AGC AAC AAC AGC GAG TGG       |
F mirr_seq_5   | CCG GAT TTA TAT CGT GGC TTC       |

Unmodified primers for RT-PCR

F ara_seq_4    | AAA AAC CAG CTA GGC CGG GA        |
F caup_seq_5   | AAC AGC AGC AGC AGC AGC A         |
F mirr_seq_4   | GAA AGC AAC AAC AGC GAG TGG       |
V5             | GAC CGG TAC GCG TAG AAT CGA G     |
DtubulinF | CGA TGC CAA GAA CAT GAT GG  
DtubulinR | GAT CGT TCA TGT TGC TCT CG  

**5’ phosphorylated primers**  
F_ara_delT_phos | AAG AAG AAT CCG TAT CCC ACA AA  
R_ara_delT_phos | GTG CTC ATT TAG CCA GGC CTT  

**Primers with restriction enzyme cleavage sites for PCR**  
F/Caup HD BamHI | CCGATCTTAC GGACCCAACT ATGATC  
R/Caup HD Xho | CCGCTCGAGT CACGGCTCCC ACGTCACTTT GTT  
intrонC_F | TGT GCT CGG CAA CAG CAT GCT GCA GC  
intrонC_R | CCG TAG ACG AAG CGC CTC TAT TTA TAC  
F_mirr_KpnI | GGG GTA CCA TGA CAG TGC ACA GTA A  
R_mirr_SacI | CGA GCT CTC ATG GCT TTG CAC TCA  
F_ara_delHD (EcoRI) | CGG AAT TCA TAG CGA AAT GGC TGC CTA CA  
R_ara_delHD (XbaI) | GCT CTA GAC TAT ATC CGA TTA GTT GGT CA  
F_cau delHD_EcoRI | GGA ATT CCA TGA CAG TGC ACA GTA A  
R_cau delHD_XbaI | GCT CTA GAA ACC GGT TCA AGG TCG AA  
F_mirr delHD_EcoRI | GGA ATT CGG TGC ATC GTA ACT CTA AGA  
R_mirr delHD_XbaI | GCT CTA GAT GCC GCC GAT GGA TG  

### 2.1.10 Fly stocks

<table>
<thead>
<tr>
<th>Stock label</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Canton S</strong></td>
<td>Bloomington Drosophila Stock Center (BDSC)</td>
</tr>
</tbody>
</table>

**Mutants**  

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Df(3L)iro-2, Sb[sbd-2]</td>
<td>BDSC (#4507)</td>
</tr>
<tr>
<td><em>iro</em>^{DPM4}</td>
<td>From S. Campuzano (Gomez-Skarmeta et al., 1996)</td>
</tr>
<tr>
<td><em>mirr</em>^{#48}</td>
<td>From H. McNeill (McNeill et al., 1997)</td>
</tr>
<tr>
<td><em>dpp</em>[d6]</td>
<td>BDSC (#2062)</td>
</tr>
<tr>
<td><em>w[</em>]; P(ry[+t7.2]=neoFRT)c82B pnr[ VX6]</td>
<td>BDSC (#6334)</td>
</tr>
<tr>
<td><em>tin</em>^{#6}</td>
<td>From M. Frasch (Azpiazu and Frasch, 1993)</td>
</tr>
<tr>
<td>Df(3L)DocA</td>
<td>(Reim et al., 2003)</td>
</tr>
<tr>
<td><em>w[</em>]; tup[isl-1] P(ry[+t7.2]=neoFRT)40A</td>
<td>BDSC (#36502)</td>
</tr>
</tbody>
</table>

**Gal4- Driver Lines**  

<table>
<thead>
<tr>
<th>Line</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>69B-Gal4</td>
<td>(Brand and Perrimon, 1993)</td>
</tr>
<tr>
<td>twi-Gal4; 24B-Gal4</td>
<td>From R. Bodmer (Klinedinst and Bodmer, 2003)</td>
</tr>
<tr>
<td>da-Gal4</td>
<td>BDRC (#8641)</td>
</tr>
<tr>
<td>twi-Gal4; Df(3L)iro-2, Sb[sbd-2]</td>
<td>generated during this work</td>
</tr>
<tr>
<td>twi-Gal4; hand-GFP</td>
<td>generated during this work</td>
</tr>
</tbody>
</table>

**UAS Lines**  

<table>
<thead>
<tr>
<th>Line</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UAS-ara</td>
<td>From S. Campuzano (Gomez-Skarmeta et al., 1996)</td>
</tr>
<tr>
<td>UAS-ara; Df(3L)iro-2, Sb[sbd-2]/TM3, lacZ</td>
<td>generated during this work</td>
</tr>
</tbody>
</table>
2.2 Working with *Drosophila melanogaster*

*Drosophila* is a very useful genetic system for studying diverse aspects of development. It has a short generation time, lasting only 220 hours (9-10 days) at 25°C (Roberts, 1986). The females lay many eggs, and therefore a large amount of progeny can be analyzed. Fly husbandry is simple and inexpensive. Moreover, large collection of mutant flies are commercially available. Last but not least, many tools are available to follow genetic traits and to manipulate gene expression in *Drosophila*.

2.2.1 Embryo collection

To collect *Drosophila* embryos, approximately 300 flies (200 females and 100 males) were transferred into the large empty fly vial, which served as a egg collection vial. This vial had small holes to let air in. The collection vial, containing flies, was closed with an apple juice agar plate with a small amount of yeast paste on it. Yeast paste was used to increase the yield of eggs. The collection bottle was then placed upside-down in an
incubator at 25°C to allow the flies to lay eggs on the agar plates. The plate, containing the eggs, was changed every 2 h and incubated at 29°C to age the embryos. Aging duration was calculated as reported previously (Park et al., 1996). Thus, the embryos were incubated for 3 h to reach stage 10-11, for 5 h 50 min (stage 12-14) and for 9h 10 min (stage 15-16). After the desired embryonic stage was reached, the embryos were transferred to a 4°C (fridge), where they were kept for one or two days. During this time the embryos are still alive, however their development is significantly slower. To prevent the embryos from drying, the agar plates, containing embryos, were placed on a humid paper towel in a Petri dish. Apple juice agar plates were prepared as described below:

### Agar plates for collecting *Drosophila* embryos

- Apple juice 50 ml
- ddH₂O 280 ml
- Bacto Agar 8 g
- Acetic acid 3.5 ml
- Ethanol (100%) 3.5 ml

Agar was added to the apple juice and water and the mixture was then boiled in the microwave. Afterwards, acetic acid and ethanol were added. Apple juice agar was then poured into caps of small Petri dishes.

### Yeast paste

- Dry yeast 1 package
- ddH₂O containing propionic acid 10-15 ml

#### 2.2.2 Embryo fixation

Aged embryos were washed from the apple juice agar plates into baskets using a funnel, paintbrush and the NaCl-TritonX solution. The chemical composition of all solutions, used for fly embryo fixation, is shown at the end of the present chapter. The baskets were made from a fine metal net and were inserted into the wells of a 24-well plate. The embryos were then rinsed 3-4 times (x) with NaCl-TritonX solution and were dechorionated in a 30% bleach solution for approximately 5 min. Dechorionated embryos were then rinsed 3x with NaCl-TritonX solution and 2x with ddH₂O. Afterwards the embryos were transferred into a glass scintillation bottle containing 5 ml of fixation solution and 5 ml of heptane. Heptane was added to increase the permeability of the vitelline membrane during the fixation and thereby facilitating the penetration of the fixation solution into the embryo. The embryos were fixed by vigorous shaking at 225 rpm
Materials and Methods

for 20 min. After 20 min, the fixation solution was exchanged with 5 ml of ddH$_2$O. The water was then removed and the embryos were dehydrated with 5 ml of methanol and were vortexed for 20s. Fixed and dehydrated embryos were then transferred into a 1,5 ml microcentrifuge tube, rinsed 3x with methanol and were either stored at -20°C or used immediately or for further analyses.

The above described fixation protocol is used for wild type embryos and embryos from flies that do not carry a so-called “blue” balancer chromosome. Balancer chromosomes are modified chromosomes containing multiple inversions and carrying a dominant phenotypic marker. The presence of the chromosomal inversions precludes chromosomal crossing over between homologous chromosomes. This is a nice tool to maintain mutant Drosophila fly lines. Additionally, the “blue” balancer chromosome carries the lacZ gene. The lacZ gene encodes for β-galactosidase (β-gal), which can be detected by X-Gal, since the cleavage of X-Gal by β-gal results in a blue product. Therefore, the embryos that are homozygous mutant remain white after the X-Gal treatment and can be selected (Bodmer, 1993).

Thus, after the 20 min fixation step, the embryos were incubated in the X-Gal solution. To this end, the fixation solution was replaced with 5 ml of ddH$_2$O. Afterwards, the water and heptane were removed and the residual heptane was carefully aspirated. The embryos were then left at room temperature for 2-3 min to allow the remaining heptane to evaporate, because the heptane may interfere with X-Gal staining. Next, the embryos were rehydrated in 5 ml of NaCl-Triton X solution for at least 5 min. Afterwards, the NaCl-Triton X solution was replaced with 1 ml of X-Gal staining solution containing 40 µl of X-Gal substrate. The embryos were then incubated at 37°C, an optimal temperature for β-gal activity. The duration of the X-Gal reaction varied for different fly lines (30 min – 4 h). The reaction was stopped when the embryos, carrying the “blue” balancer, were discernible blue. The X-Gal staining solution was discarded. Subsequently, 5ml of prewarmed (65°C) heptane was applied to the embryos first, followed by 5ml of prewarmed (65°C) methanol. Lastly, the embryos were vortexed for 20s, transferred into a 1,5 ml microcentrifuge tube, rinsed 3x with methanol and stored at -20°C.
## Materials and Methods

**Solutions used for fixation of *Drosophila* embryos**

<table>
<thead>
<tr>
<th>Paraformaldehyde (10%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Paraformaldehyde (PFA) 40 g</td>
</tr>
<tr>
<td>- 0.5 M Na$_3$PO$_4$, pH 7.6 48 ml</td>
</tr>
<tr>
<td>- ddH$_2$O to a total volume of 400ml</td>
</tr>
</tbody>
</table>

The solution was made by heating to 60-65°C on the magnetic stirrer, aliquoted (10-14 ml) and stored at -20 °C.

<table>
<thead>
<tr>
<th>0.5 M Na$_3$PO$_4$, pH 7.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Na$_2$HPO$_4$ x 2 H$_2$O 0.5 M</td>
</tr>
<tr>
<td>- NaH$_2$PO$_4$ x H$_2$O 0.5 M</td>
</tr>
</tbody>
</table>

The two salt solutions were made separately. pH 7.6 was obtained by combining the two solutions, Na$_2$HPO$_4$ and NaH$_2$PO$_4$. Aliquots were kept at -20°C.

<table>
<thead>
<tr>
<th>Fixation Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>- 10% PFA 2 ml</td>
</tr>
<tr>
<td>- 0.06 M Na$_3$PO$_4$ 3 ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NaCl-Triton X Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>- NaCl 7 g</td>
</tr>
<tr>
<td>- Triton X-100 0.4 ml</td>
</tr>
<tr>
<td>- ddH$_2$O to a total volume of 1 l</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>X-Gal Staining Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Na$_3$PO$_4$, pH 7.2 1.64 g</td>
</tr>
<tr>
<td>- NaCl 8.76 g</td>
</tr>
<tr>
<td>- MgCl$_2$ 0.09 g</td>
</tr>
<tr>
<td>- K$_4$[Fe(CN)$_6$]x3H$_2$O 1.27 g</td>
</tr>
<tr>
<td>- K$_4$[Fe(CN)$_6$] 1.1 g</td>
</tr>
<tr>
<td>- Triton X-100 0.3 ml</td>
</tr>
<tr>
<td>- ddH$_2$O to a total volume of 1 l</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bleach</th>
</tr>
</thead>
<tbody>
<tr>
<td>- DanKlorix 30 ml</td>
</tr>
<tr>
<td>- ddH$_2$O 70 ml</td>
</tr>
</tbody>
</table>

The solution was always prepared fresh.

To analyze phenotypes that may affect expression of the transcription factor *hand*, a *hand*-GFP reporter line was used (Sellin et al., 2006). In this case, the embryos were not fixed, because the fixation procedure does not preserve GFP-fluorescence. However, bleach dechorionation was required to make it possible to see the GFP fluorescence under the microscope. Thus, the embryos, carrying *hand*-GFP, were mounted on a glass slide in Vectashield immediately after the dechorionating step and were analyzed instantly.
2.3 Histology

2.3.1 Whole mount fluorescent immunostaining of *Drosophila* embryos

Single and double immunostainings were performed to detect the protein/s of interest in the embryo and to analyze heart phenotypes. This method is based on the principle of binding of the antibody to the antigen. The signal of some antibodies was amplified using the Tyramide Signal Amplification (TSA) Biotin System (NEL700A kit). Washing buffer composition and other solutions, used for the immunohistochemistry, are presented at the end of this chapter.

*Single immunostaining*

For a single immunostaining, fixed embryos were rinsed twice with methanol. Unless otherwise mentioned, all incubation and washing steps were done using 1 ml of a solution and carried out at room temperature on a rocker. To inactivate the endogenous peroxidase, the embryos were then incubated for 20 min in methanol containing 1,5% hydrogen peroxide. The presence of endogenous peroxidase in the embryos may result in a nonspecific background staining, since the amplification of the antibody signal with the TSA kit includes a step with Horseradish peroxidase (HRP) conjugated Streptavidin (SA). After inactivating the endogenous peroxidase, the embryos were washed twice with methanol, once with a methanol:TNT buffer mixture (1:1) and 3x with TNT buffer, each time for 5 min. To prevent unspecific binding of the antibody, the embryos were then incubated in the blocking solution (TNB) for at least an hour. The blocking solution was replaced with 200µl of the desired primary antibody diluted in TNB. The embryos were then incubated in the primary antibody solution overnight at 4°C on a rocker.

The next day, the embryos were washed 8x with TNT buffer, each time for 15 min. The antibody signal was then amplified using the TSA kit, which is based on the high binding affinity of Biotin to Avidin. Therefore, the embryos were incubated with a biotinylated secondary antibody diluted in TNB (1:200) for 2 h. Afterwards, specimens were washed 6x with TNT buffer, each time for 15 min. The embryos were then incubated in 200µl of SA-HRP (Streptavidin coupled to Horseradish peroxidase from the TSA kit) solution, diluted in TNB (1:100) for 30 min, followed by 4x TNT washes, for 15 min each. The second amplification of the signal was done by incubating the embryos in 50µl of a 1:50 dilution of Biotin-Tyramid Reagent in the Amplification solution (from the TSA kit)
for 5 min on the bench. Afterwards, the embryos were washed 4x with TNT buffer for 15 min and were incubated in 200µl of SA-DTAF:TNT (1:200) for 30 min. DTAF (dichlorotriazinylamino fluorescein) is a fluorophore that makes the antibody signal visible by binding to the Biotin-protein complex. Lastly, the embryos were washed 6x with TNT buffer for 15 min. The embryos were then mounted on a glass slide using Vectashield mounting media and were covered with a coverslip. To prevent the slides from drying, the edges of the coverslip were sealed with a nailpolish.

Double immunostaining

For a double immunostaining, the signal of the first primary antibody was amplified using the TSA system, whereas the signal of the second primary antibody was not amplified. When the two primary antibodies used for the double immunostaining were generated in different animal species, the second primary antibody was added to the embryos immediately after the final 6 washing steps of the single immunostaining protocol. The embryos were then incubated in the second primary antibody solution on the rocker overnight at 4°C. If both primary antibodies originate from the same animal species, stripping of the first primary antibody was required. To this end, the embryos were washed 3x with a stripping solution, for 10 min each, followed by 4x TNT washes (15 min) and were then incubated in the second primary antibody solution (200µl) overnight at 4°C. The next day, the specimens were washed 8x with TNT buffer for 15 min and incubated with the secondary antibody (diluted 1:200 in TNB) for 2 h at room temperature (or overnight at 4°C). To this end, it is necessary to use a secondary antibody that is conjugated to a fluorophore. In current work, secondary antibodies that are coupled to either Cy3 or to DyLight594 were used. Finally, the embryos were washed 6x with TNT buffer for 15 min and were mounted on a slide.
Materials and Methods

Solutions for whole mount immunohistochemistry

<table>
<thead>
<tr>
<th>Stock solutions</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris/HCl, pH 7,5</td>
<td>1M</td>
</tr>
<tr>
<td>NaCl</td>
<td>5M</td>
</tr>
<tr>
<td>TNT buffer</td>
<td></td>
</tr>
<tr>
<td>1M Tris/HCl, pH 7,5</td>
<td>0.1M</td>
</tr>
<tr>
<td>5M NaCl</td>
<td>0.15M</td>
</tr>
<tr>
<td>Tween 20</td>
<td>0.05%</td>
</tr>
<tr>
<td>TNB buffer</td>
<td></td>
</tr>
<tr>
<td>1M Tris/HCl, pH 7,5</td>
<td>0.1M</td>
</tr>
<tr>
<td>5M NaCl</td>
<td>0.15M</td>
</tr>
<tr>
<td>Blocking reagent (from the TSA Kit)</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

The Tris/NaCl buffer was heated to 60°C using a water bath, on a magnetic stirrer and the blocking reagent was then slowly added. Aliquots were stored at -20 °C.

Stripping Solution

<table>
<thead>
<tr>
<th>Stripping Solution</th>
<th>100ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine/HCl, pH 2,5</td>
<td>250 mM</td>
</tr>
<tr>
<td>Tween80</td>
<td>0.1%</td>
</tr>
</tbody>
</table>

All solutions are water based and ddH₂O was used.

2.3.2 Whole mount fluorescent in situ hybridization and immunostaining

A fluorescent in situ hybridization was performed to visualize mRNA transcripts using Digoxigenin (DIG) labeled antisense RNA. Solutions and buffers used for in situ hybridization are listed at the end of this chapter.

The dpp and pnr in situ probes were generated using the previously published plasmids (Padgett et al., 1987; Ramain et al., 1993). The plasmids containing ara and caup cDNAs were obtained from the DGRC. The ara plasmid was linearized using the BglIII restriction enzyme, which has a cleavage site internal to the ara ORF. The in vitro transcription of ara in situ probe from the linearized plasmid resulted in a RNA fragment containing 3’UTR region and 758bp of ara ORF. A 982bp sequence of caup ORF was cut from the plasmid containing caup cDNA using EcoRI and HindIII enzymes and subcloned into the pBlueScript II KS (+) vector. Subsequently, the plasmid was linearized using the EcoRI and the antisense RNA was synthesized. As for the in situ probe for mirr, a 930bp of mirr ORF was amplified by PCR, cloned into the pCR4-TOPO vector, linearized using the NotI enzyme and transcribed in vitro.

Labeling and the in vitro transcription of all in situ probes was done using the DIG RNA Labeling mix and the T3 RNA polymerase according to the manufacturer’s
instructions. *In situ* probes were purified using the MicroSpin G-50 columns (GE Healthcare), according to the manufacturer’s protocol.

**In situ hybridisation**

Fixed *Drosophila* embryos were rinsed twice with methanol. All rinsing, washing and incubation steps were done using 1 ml of solution, at room temperature, on a rocker, unless otherwise noted. The endogenous peroxidase was inactivated by incubation of specimens in methanol containing 1.5% hydrogen peroxide for 20 min and the embryos were then washed twice with methanol and subsequently with a methanol:xylene mixture series (3:1, 1:1 and 1:3) for 5 min each. The methanol:xylene mixture was then replaced by 500 μl xylene. When xylene is added, it is important not to lose the embryos, because xylene treatment makes them transparent. Next, the fly embryos were incubated in 1.5 ml of xylene for an hour. After this incubation step, the specimens were rinsed once with a methanol:xylene (1:1) mixture, 3x with methanol, 1x with methanol:PTW buffer (1:1) and 3x with PTW buffer. The embryos were then fixed 3x in 2% formaldehyde solution (diluted in PTW), for 3 min, 30 min and 20 min. After each fixation step, the specimens were rinsed 3x with PTW. The embryos were then rinsed once with PTW:pre-hybridization solution mixture (1:1) and 2x with pre-hybridization solution. The pre-hybridization solution must be prewarmed to room temperature before use. Afterwards, the embryos were incubated in 800 μl of pre-hybridization solution for 2-3 h at 53°C. Next, 2-4μl of DIG-labeled antisense RNA probe were heated in 100 μl of hybridization solution at 95°C for 5 min and were then cooled down on ice for 1-2 min. The pre-hybridization solution was replaced by the DIG-labelled RNA probe (*in situ* probe) containing solution. The hybridization was performed for at least 36 h since the proteinase K treatment was omitted.

After 36 h, 800μl of prewarmed (53°C) pre-hybridization solution was added directly to the embryos onto the *in situ* probe solution and was then incubated for 20 min. While the embryos were incubating, different ratios (4:1, 3:2, 2:3 and 1:4) of pre-hybridization:PTW mixtures were prepared and preheated at 53°C. The pre-hybridization:PTW mixtures were subsequently added to the embryos and incubated each time for 20 min at 53°C. The specimens were then incubated in prewarmed PTW for 10 min at 53°C. Afterwards, the embryos were rinsed twice with PTW at room temperature and were further incubated in 500μl of RNase A (Roche) solution (diluted in PTW 1:500)
for 20 min at 37°C. Subsequently, the embryos were rinsed 3x with PTW and incubated with Sheep-anti-DIG antibody, diluted in PTW 1:1000, overnight at 4°C. The next day, the embryos were washed 8x with TNT buffer, 15 min each and were incubated with biotinylated anti-sheep secondary antibody (1:200, diluted in TNB) for 2 h. The TSA Biotin System (Perkin Elmer) was used to amplify the signal of the labeled mRNA probe. Therefore steps following the incubation with a biotinylated secondary antibody were identical to those described in the fluorescent immunostaining protocol (see chapter 2.3.1).

**Fluorescent in-situ hybridization coupled with immunostaining**

After completing the in-situ hybridization, i.e. after the final 6 washes with TNT, the desired primary antibody was applied to the embryos and incubated overnight at 4°C. Subsequently, the procedure was continued as it is described for the double immunostaining (see chapter 2.3.1), starting from the step when the second primary antibody is applied.

### Solutions for whole mount in situ hybridization

#### 10 x PBS (pH 7.4; 1l)

- NaH₂PO₄ x H₂O 2.56 g
- Na₂HPO₄ x 2H₂O 14.96 g
- NaCl 102.2 g

The solution is water based and DEPC treated ddH₂O was used.

#### PTW (500 ml)

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An autoclaved ddH₂O was added and stored in the fridge.

#### Pre-hybridization solution (250 ml)

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<td>Denhardt’s</td>
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DEPC treated ddH₂O was used to make the solution and it was stored at -20°C.

#### Hybridization solution (50 ml)

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<td>Torula RNA</td>
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Stored at -20°C.
2.4 Cell culture methods

2.4.1 Maintenance of Drosophila Schneider 2 (S2) cells

_Drosophila_ S2 cells were cultured in 25cm² T-flasks containing 5 ml of Schneider's _Drosophila_ Medium supplemented with 9% fetal bovine serum (FBS) at 25ºC. For maintenance of S2 cells, the cells were split at a 1:5 dilution every 2-3 days. For example, 1 ml of the cell suspension (approximately 20x10⁶ cells/ml) were transferred into a new flask containing 4 ml of fresh medium.

2.4.2 Dual-Luciferase Reporter Assay

A binding site for Caup, 5’ACAATAACT3’, was indentified on the previously published enhancer region of _hand_ (Han and Olson, 2005; Sellin et al., 2006). To examine whether Iro-C members may regulate the transcription of _hand_ in vitro, the _hand_-luciferase reporter construct (_hand_-luc) was generated by cloning the 513bp regulatory sequence of _hand_ into the luciferase vector pGL4.26luc2.

*Generation of ara-pAc5.1, caup-pAc5.1C and mirr-pAc5.1C constructs (for transfection)*

The complete ORFs of _ara_ and _caup_ were isolated by PCR from the _ara_ and _caup_ containing plasmids (obtained from the DGRC) and were subcloned into pAc5.1C/HisC vector. The complete ORF _mirr_ was also amplified via PCR from the plasmid containing the _mirr_ cDNA (a kind gift from H. McNeill) and was subcloned into pAc5.1C/HisC vector. The forward and the reverse primers used for these PCR reactions contained extra base pairs at the 5’ end, the EcoRI and the XbaI restriction enzyme recognition sites respectively, which allowed the subsequent cloning into the corresponding sites in the pAc5.1C/HisC vector.

*Transfection of S2 cells*

400µl of cell suspension (1,25 x 10⁶ cells/ml) were pipetted into each well of a 24-well plate. The cells were then incubated for at least 2 hours at 25ºC to adhere to the bottom of the wells. After 2 h, S2 cells were transfected with 20ng of Renilla luciferase reporter plasmid (Rluc), as a transfection control, (kindly provided by H. Ueda)
(Matsumoto et al., 2007), 100ng of hand cardiac enhancer in the luciferase vector and 300ng of either ara, caup or mirr cDNA. Transfection was performed using FectoFly DNA transfection reagent for insect cells according to the manufacturer’s protocol. The transfected cells were then incubated at 25°C for 24 h.

Measurements of luciferase activity

The cells were lysed after 24 hours using the Dual-Luciferase Reporter Assay System according to the manufacturer’s instructions. Most of the cell lysate (90µl out of 100µl) was transferred from the 24-well plate into a 1,5ml microcentrifuge tube and was then stored at -70°C for measuring luciferase activity later. The remaining 10µl of the lysate were used instantly to isolate RNA (2.5.1) that was employed for cDNA synthesis and subsequent PCR to verify expression levels of the transfected constructs for either ara, caup or mirr. In order to determine whether the MAPK pathway might affect the hand-luc activation by Ara, Caup and/or Mirr, S2 cells were treated with 2µl of MAPK inhibitor PD098059 (6.5mg/ml, diluted in DMSO) 2 hours before lysing cells. As a control, cells were transfected with the same constructs and treated with 2µl of DMSO.

Luciferase activity was measured using a luminometer. The Luciferase Assay Reagent II (LAR II) and Stop&Glo were prewarmed to room temperature. 50µl of LAR II and 10µl of cell lysate were mixed by pipetting up and down in the luminometer vial and the luciferase activity was then measured. Subsequently, 50µl of Stop&Glo reagent were added to the mixture after approximately 10 sec and the Rluc activity was then measured. Luciferase activity was normalized to Rluc activity. Four or more independent experiments were performed, each was done in triplicates. The data was analyzed statistically with the Mann-Whitney test (GraphPad Prism software).

2.5 Molecular biology methods

2.5.1 RNA isolation from the S2 cells

To isolate RNA from S2 cell lysates (see 2.4.2) the RNA-magic (phenol-chloroform based) reagent was used. To this end, 10µl of cell lysate (from each transfection) were transferred from the 24-well plate into a 1,5ml microcentrifuge tube. As the luciferase reporter assay was performed in triplicates, cell lysates of 3 wells transfected
with the same construct were pooled. Thus, 30µl of cell lysate were used in total for RNA extraction. 300µl of RNA-magic were added to the 30µl cell lysate, mixed by brief vortexing and the mixture was incubated for 5 min at room temperature. After 5 min, 100µl of chloroform were added and the mixture was vortexed for 10 sec. Specimens were then incubated on ice for 10 min. Starting from this step, samples were kept on ice or were centrifuged at 4ºC, unless otherwise noted. After 10 min, the mixture was centrifuged 10 min at 13000rpm. Centrifugation helps to separate the organic phase from the aqueous phase. The upper aqueous phase, containing the RNA, was then transferred into a new sterile 1,5ml microcentrifuge tube and an equal amount of isopropanol was added to precipitate the RNA by incubation on ice for 15 min. Samples were then centrifuged for 10 min at the highest speed. The liquid was discarded and the RNA precipitate was washed 3x with 70% ethanol. The first two washes were followed by centrifugation at the highest speed for one minute. After the 3rd wash, samples were centrifuged for 10 min, the ethanol was discarded and the RNA pellet was left at room temperature for 5-10 min to evaporate residual ethanol. Afterwards the RNA pellet was resuspended in 20-30µl of nuclease-free water.

To eliminate DNA contamination, the RNA was treated with DNaseI at 37ºC for 15 min. Afterwards, the RNA concentration was measured using a NanoPhotometer. 100ng of the RNA were used for the cDNA synthesis using Superscript III First-Strand Synthesis System for the reverse-transcription-PCR (RT-PCR) according to the manufacturer’s instructions.

### 2.5.2 Polymerase chain reaction

For the RT-PCR, GoTaq PCR kit and Phire Hot Start II PCR Master Mix kits were used. The RT-PCR reaction mixture and the programs were set up according to the kit protocol. To amplify desired DNA fragments used for cloning the Pfu Ultra II Fusion HS DNA-polymerase was used. The reaction mixture was set up in a PCR tube as follows: 40,5µl of H2O, 1µl (10pmol/µl) of the forward and 1µl (10pmol/µl) of the reverse primer, 5µl of Pfu Ultra Reaction Buffer, 0,5µl of dNTPs (25mM), 1µl of Pfu Ultra DNA Polymerase and 1µl of the template DNA. Standard PCR program was used, including initial denaturation at 98 ºC for 2 min followed by 30-35 cycles of denaturation at 98ºC for 20 sec, annealing at 50-55ºC for 20sec, elongation at 72ºC for 30sec/kb and the final elongation was done at 72ºC for 7 min.
When primers carrying restriction enzyme recognition sites were used, the PCR program was modified. The initial denaturation was carried at 98°C for 2min, followed by 5 cycles of denaturation at 98°C for 20 sec, calculated annealing temperature of the primers without cleavage ends for 20 sec, elongation at 72°C for 15sec/kb. Subsequently, the reaction was continued with 30 cycles as described above.

PCR products were then analyzed on a 0.8-1% agarose gel containing ethidium bromide. The size of the DNA band was determined by use of DNA-gene ruler.

2.5.3 DNA restriction digest and ligation

Restriction enzyme digestion was performed using suitable enzymes and buffers, according to the manufacturer’s instructions (BioLabs). For cloning of a PCR product (see 2.5.2) into a vector, the cDNA fragment to be inserted and the vector were digested separately using same restriction enzymes. Afterwards, the two products of digestion reaction were purified from a 1% agarose gel separately using GeneJET Gel Extraction Kit, according to the manufacturer’s instructions and were eluted in 20-30µl of elution buffer. Insertion of the DNA into the vector was done using Ligate-IT Rapid Ligation Kit, according to the manufacturer’s protocol. Plasmids were then used for subsequent transformation or kept at -20°C.

2.5.4 DNA transformation and plasmid DNA purification

DNA transformation was done using E. coli One Shot TOP 10 chemically competent cells according to the manufacturer’s protocol. The resulting suspension of transformed bacteria was then poured on a prewarmed LB-agar plate containing an appropriate antibiotic (see below). The plate, containing bacteria, was incubated overnight at 37°C. The next day, a single colony of bacteria was used to inoculate a liquid LB-medium (see below), containing 100µg/ml of ampicillin, for 12-16 hours at 37°C, continuously shaking at 220rpm.
Plasmids were purified using either the QIAfilter Midi plasmid purification kit or the Wizard Plus SV Minipreps DNA Purification System, according to the manufacturer’s instructions and were verified by sequencing.

2.5.5 Sequencing probe preparation

For sequencing, the DNA fragment of interest was amplified by PCR using DYEnamic ET Terminator Cycle Sequencing Kit. Sequencing primers were designed for every 400-450 nucleotides of the insert DNA sequence. The reaction mixture was prepared as follows: 0,5ng of plasmid DNA, 1 µl of primer (3,3 pmol/µl), 4 µl of the DYEnamic kit mixture and a nuclease free water to a total volume of 10µl. The PCR was done for 30 cycles with a denaturation step at 95ºC for 20 sec, annealing at 50ºC for 15 sec and the elongation at 72ºC for 60 sec. Afterwards, the PCR product was purified from the reaction mixture. To this end, amplified DNA fragment was transferred into a 1,5ml microcentrifuge tube and 1µl of 1,5M sodium acetate (pH 7,5) and 40 µl of ethanol were added to precipitate the DNA. The mixture was then vortexed briefly and incubated on ice for 20 min. After 20 min, the sample was centrifuged 15 min at the highest speed at 4ºC. Subsequently, the liquid was discarded and the precipitated DNA was then washed twice with 200µl of ice-cold 70% ethanol. Each wash was performed by centrifuging for one minute at 4ºC. Lastly, the sample was left at room temperature for 5-10 min to evaporate residual ethanol and ready for use in a sequencer. The DNA sequence analyses were performed using the SerialCloner 1-3 software.

2.5.6 Electrophoretic Mobility Shift Assay (EMSA)

To test whether Caup binds to the hand enhancer region, a gel electrophoresis mobility shift assay (EMSA) was performed. In this assay, solutions of $\gamma^{32}$P-labeled
nucleic acid and protein are combined and the resulting mixture is subjected to electrophoresis, using a non-denaturating polyacrylamide gel. The results are analyzed by autoradiography of $\gamma^{32}$P-labeled DNA fragment.

Preparation of the protein

The homeodomain (HD) of caup was cloned into a pGEX-4T-1 vector (containing a GST-tag) and the insert was validated by sequencing. To obtain the GST-CaupHD protein, BL21 competent cells were transformed with the GST-CaupHD plasmid, according to the manufacturer’s instructions. Afterwards, the protein was purified using the Glutathione Sepharose-4B as it is described in the manufacturer’s protocol. The protein was then dialyzed using the D-Tube Dialyzer Midi overnight on a magnetic stirrer at 4°C, in the dialyzing buffer (see below). The GST-CaupHD fusion protein (35 kDa) was then subjected to electrophoresis on a 12% polyacrylamide gel and verified by Cooomassie staining according to the standard protocol, and the probe was then ready for use.

Labelling of the oligonucleotide probe (putative Caup-binding site in the enhancer region of hand)

The sense, 5’TTG GTT TGT TAT TGA AAG CAT3’, and the antisense, 3’ATG CTT TCA ATA ACA AAC CAA5’, oligonucleotides, containing the predicted Caup-binding site on the hand enhancer, were each labelled with $\gamma^{32}$P ATP using the T4 Polynucleotide Kinase kit, according to the manufacturer’s instructions. Afterwards, labeled oligonucleotides were combined and incubated for 5 min at 95°C and were left in the turned off heating block for 2-4 hours for annealing. The labelled double stranded DNA was then purified using the G-50 column and was used for the binding reaction.

Binding reaction

The total volume of the binding reaction was 30µl. For this reaction, 17µl of GST-CaupHD, 2µl of the labeled DNA probe, 1µl (1µg/µl) of poly dIdC (used to prevent unspecific binding), 1,5µl of NP-40, 6µl of 5x binding buffer (Bilioni et al., 2005) and 2,5µl of ddH$_2$O were combined in a microcentrifuge tube and incubated for 15 min on ice.

For the competition test, either additional 2,5µl (1µg/µl) of poly dIdC were added, or 2,5µl (500pmol/µl) of unlabeled DNA (so called “cold” probe) were added to the binding reaction (instead of 2,5µl of water). To exclude that the signal could result from
Materials and Methods

GST binding to the putative Caup binding site, the binding reaction was set up using only the GST protein.

1/3 of each binding reaction was then loaded into the wells of a 8% nondenaturating polyacrylamide gel and the electrophoresis was performed at 215 V (in a 0.5x TBE buffer) at 4°C. After electrophoresis, the gel was exposed to imaging plate overnight at 4°C and analyzed using the BAS100 Phosphoimager (Fujifilm).

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3 Results

3.1 Embryonic expression pattern of the members of the *Drosophila Iroquois* complex

The expression pattern of Ara, Caup and Mirr was analyzed during embryogenesis with respect to their possible involvement in heart development. The focus of the current work is the mesodermal expression of *ara, caup* and *mirr*, since the heart of *Drosophila* originates from the mesoderm. To this end, a polyclonal anti-Caup antibody was used, which is the only available antibody against Caup (Carrasco-Rando et al., 2011; Diez del Corral et al., 1999; Letizia et al., 2007). However, this antibody detects Ara as well and therefore the immunostainings are labelled as Ara/Caup. At embryonic stage 10, Ara/Caup proteins are expressed in an undulating pattern in the dorsal mesoderm (Figure 3 A). At this stage, these proteins are also detected along the dorsal edge of the ectoderm (Figure 4 C and Calleja et al., 2000; Carrasco-Rando et al., 2011; Letizia et al., 2007). By stage 11, Ara/Caup expression becomes restricted to segmental patches adjacent to Eve cell clusters (Figure 3 B). Eve marks clusters of cells that give rise to a subset of pericardial cells and DA1 dorsal muscle cells (Carmena et al., 2002; Frasch et al., 1987; Su et al., 1999).

Double labelling for *caup* mRNA and Tin protein further confirms the presence of *caup* transcripts in the dorsal mesoderm that encompasses cardiac and visceral mesoderm (Figure 3 C). An *in situ* hybridization for *ara* mRNA transcripts showed a similar pattern however *ara* mRNA transcripts are present at a lower level compared to the mesodermal expression of *caup* mRNA transcripts (Figure 4 A compared to B and Carrasco-Rando et al., 2011). At stage 11, the initially broad Tin expression segregates into two clusters of cells per segment, cardiac precursors (dorsal cell cluster) and visceral mesoderm cells (ventral cell cluster) (Lockwood and Bodmer, 2002). At this stage, *caup* mRNA expression is stronger around the Tin-positive cells in the visceral mesoderm compared to cardiac mesoderm (Figure 3 D). During stage 12, Ara/Caup expression disappears from the dorsal domain of the embryo, where the heart progenitors have been specified and begin to differentiate (Figure 3 E). Ara/Caup expression is detectable again during stage 13 in single cells along the Dmef2-positive myocardial cell rows (Figure 3 F). At stage 15, Ara/Caup is present in single cells located in a segmental pattern between the Eve-expressing pericardial cells (Figure 3 G). In addition, double labelling for Ara/Caup and
the pericardial marker Eve demonstrates that Ara/Caup is co-expressed with Eve at the anterior tip of the heart. To determine the exact position of Ara/Caup-positive cells, located along the dorsal vessel, a double immunostaining for Ara/Caup and β-Tubulin was performed. β3-Tubulin is a structural protein that is expressed in diverse muscle cells, including myocardial cells. Its expression in the heart is limited to four out of the six myocardial cells per hemisegment that also express Tin (Leiss et al., 1988).

**Figure 3. Wildtype expression pattern of Ara/Caup and Mirr during cardiogenesis.**

(A) At stage 10, Ara/Caup are expressed in the mesoderm along the dorsal side of the embryo. (B) Slightly later Ara/Caup cells are arranged segmentally in close proximity to the Eve-expressing clusters. (C) The mesodermal expression of *caup* is shown by double labelling for Tin protein and *caup* mRNA at stage 10. (D) By stage 11, *caup* mRNA is still present in an overlapping manner with Tin, whose expression has been segregated to cardiac progenitors (arrows) and cells of the visceral mesoderm (asterisks). The insets in C and D are higher magnifications showing co-localization of Tin (red) and *caup* transcripts (green). (E) At stage 12, Ara/Caup are no longer detected in the cardiogenic region (asterisks). (F) Starting at mid embryogenesis, Ara/Caup-positive cells are detectable again along the myocardial cell row that expresses Dmef2 (arrows). Arrows point to Ara/Caup cells located between the Eve pericardial cells. (G) Ara/Caup is co-expressed with Eve in the anteriorly located Eve-positive cells at stage 15 (arrowheads). (H) The single Ara/Caup-expressing cells (arrows) are located laterally to β3-Tubulin-expressing myocardial cells. Arrowheads point to the two cells that are negative for β3-Tubulin. (I) Lateral view of a 10/11 stage embryo showing Mirr expression in the dorsal mesoderm. (J) At stage 11 Mirr-positive cells start to be clustered around the Eve-expressing cells. (K) At stage 16, pairs of Mirr cells are detected between the Eve-expressing pericardial cells (arrows). (L) One of these Mirr-positive cells co-expresses Ara/Caup (arrowhead) at late embryonic stages, whereas the other is positive for Mirr only (arrows). (A-F, I, J) Lateral views of embryos; (G, H, K, L) dorsal views of embryos. In all panels anterior is towards the left.
At embryonic stage 16, Ara/Caup-positive cells are located laterally to β-Tubulin expressing myocardial cells and consequently also laterally to the Tin-positive myocardial cells (Figure 3 H).

The expression pattern of the third member of the Iro-C, Mirr, is similar to that of Ara/Caup at stage 10/11 and mirr transcripts are detected in the dorsal mesoderm (Figure 3 I) as well as in the dorsal ectoderm (Figure 4 D and McNeill et al., 1997). During stage 11, the initial continuous expression of Mirr becomes arranged in segments in the mesoderm, where the Mirr-positive cells surround Eve-expressing clusters (Figure 3 J). Subsequently, Mirr expression disappears from the dorsal mesoderm during stage 13-14 (data not shown) and it appears again in pairs of cells along the fly heart in a segmentally repeated pattern at stage 16 (Figure 3 K). In one of the two Mirr expressing cells in each hemisegment, Mirr is co-expressed with Ara/Caup (Figure 3 L). Unlike Ara/Caup (Figure 3 G), Mirr is not co-expressed with Eve in any of the Eve-positive pericardial cells.

Figure 4. Wildtype expression pattern of ara and caup transcripts in the mesoderm and ectodermal expression of the members of Iro-C.
(A) At stage 10, ara mRNA is detected in the mesoderm lateral to Eve-positive cell clusters (arrows). (B) At this stage, caup transcripts are expressed similar to ara (arrows), although caup mRNA is expressed at higher levels compared to ara (compare B to A). Asterisks in A and B point to ara and caup mRNA staining in the amnioserosa. (C) At stage 10, double labelling for Ara/Caup and Engrailed (En) shows that co-localized Ara/Caup and En in the dorsal ectoderm appear yellow. (D) Stage 10 embryo showing overlapping expression of mirr mRNA and En protein in dorsal ectoderm. Lateral views of embryos are shown.

To determine whether the Ara/Caup and/or Mirr expressing cells that are located in the vicinity of the Eve pericardial cells (Figure 3 G, K) belong to one of the known pericardial cell types, Ara/Caup and Mirr expression was compared with the expression of various pericardial markers, such as Tin, Odd, Zfh-1 and with the expression of Pericardin (Prc), extracellular matrix protein that is located around the pericardial cells (Chartier et
Analyses of double immunostainings for Ara/Caup or Mirr and one of the mentioned pericardial markers demonstrated that Iro-C members are not co-expressed with any of the factors tested (Figure 5).

**Figure 5. Ara, Caup and Mirr identify a new subset of heart-associated cells.**
(A) At stage 15, Ara/Caup cells (arrows) are located between Tin-positive pericardial cells (arrowheads). (B) Ara/Caup (arrows) and Odd are not co-expressed in Odd-positive pericardial cells. (C) caup mRNA does not co-localize with the pericardial marker Zfh-1. (D) Mirr-expressing cells (arrows) are distinct from Zfh-1-positive pericardial cells. (E) Mirr-positive cells (arrows) do not express Tin. (F) Mirr (arrows) and Prc expression do not overlap. The insets in D and F are higher magnifications for better illustration. Dorsal views of embryos are shown; anterior is to the left.

In summary, the early expression of *ara, caup* and *mirr* at stage 10/11 in the dorsal mesoderm suggests a role for *Iro-C* in determining territories with different cell fates. The co-localization of Ara/Caup and Eve in the anterior Eve-positive pericardial cells proposes a function for Ara/Caup in the diversification of pericardial cells. Finally, the Ara/Caup and Mirr-positive cells, detected adjacent to the forming heart, may represent a novel group of heart-associated cells.
3.2 The Iroquois complex is required for normal expression of the crucial cardiac transcription factors Tup, Doc2 and Tin but does not affect the expression of pnr

In Drosophila the mesodermal expression pattern of Ara/Caup and Mirr at early stages suggests a role for these factors in patterning the dorsal mesoderm. Consequently Iro-C may affect the proper development of mesoderm derivatives including the heart. In the current work, the role of Iro-C in Drosophila heart development was characterized using the Df(3L)iro-2, iro\textsuperscript{DFM1} and mirr\textsuperscript{e48} embryos. The Df(3L)iro-2 fly line harbours a large chromosomal deletion including ara, caup and mirr (Gomez-Skarmeta et al., 1996; Kehl et al., 1998; Leyns et al., 1996). The iro\textsuperscript{DFM1} fly line lacks ara and caup but still expresses mirr (Gomez-Skarmeta et al., 1996; Kehl et al., 1998; Pichaud and Casares, 2000). The mirr\textsuperscript{e48} carries a 1kb deletion in the promoter region of mirr and therefore the mirr\textsuperscript{e48} embryos are mutant for mirr only (McNeill et al., 1997; Zhao et al., 2000). To confirm the effect of lack of ara and caup on heart development, embryos with the Df(3L)iro-2/iro\textsuperscript{DFM} genetic background were analyzed. These embryos are homozygous mutant for ara and caup and heterozygous for mirr.

Tin, pnr, tup, and Doc are well-characterized transcription factors that have been demonstrated to be important for different steps of dorsal vessel development. Specification of cardiac mesoderm and formation of heart progenitors are critically dependent upon the orchestrated expression of these factors. To determine the effect of lack of Iro-C on cardiac mesoderm formation as well as on heart cell specification (at stage 10/11), Tup, Doc and Tin protein and pnr mRNA expression was examined in the embryos that are mutant for the entire Iro-C (Df(3L)iro-2), in ara/caup mutants (iro\textsuperscript{DFM1}), in heterozygous Df(3L)iro-2/iro\textsuperscript{DFM} embryos (Figure 6) and in mirr mutants (mirr\textsuperscript{e48}) (Figure 7). The quantification of phenotypes in all investigated mutants is shown in the histograms in Figure 6 Q-T.

All tested mutants show a reduction of Tup-positive cells in the cardiac mesoderm (Figure 6 A-D and Figure 7 A, B). In the embryos that lack all three members of Iro-C (Df(3L)iro-2) the penetrance of the Tup phenotype was higher (72%) in comparison to the penetrance of 19-27% in the other investigated mutant embryos (Figure 6 Q). Doc expression was strongly reduced in the embryos that lack all three Iro-C members and in
the embryos that are mutant for *ara* and *caup* (Figure 6 E-H). Some of the *mirr*°48 embryos were also characterized by a reduction of Doc-expressing cells (Figure 7 C, D), however the penetrance of the Doc phenotype in *Df(3L)iro-2*, in *iroDFM1* and in *Df(3L)iro-2/iroDFM1* embryos was higher in comparison to the penetrance of the phenotype in *mirr* mutants (Figure 6 R). In addition, the severity of the Doc2 phenotype in the embryos that lack only *ara/caup* or only *mirr* also differed, with Doc2 expression being only mildly reduced in *mirr* mutants (Figure 7 C, D). These findings suggest that *mirr* might be less important for maintaining Doc expression. In contrast to the phenotype observed for Tup and Doc2, the majority of all analyzed mutants showed normal expression of *pnr* transcripts (Figure 6 I-L, S and Figure 7 E, F). Unchanged *pnr* mRNA expression in these mutants was unexpected because it is known that *Doc* and *tup* expression require the presence of *pnr* (Mann et al., 2009; Reim and Frasch, 2005). However, in spite of normal *pnr* mRNA expression, Doc- and Tup-expressing cells were reduced in *ara/caup* mutants. This finding is interesting because it is possible that during cardiogenesis *pnr* may cooperate with *Iro-C* members to maintain the expression of Doc and Tup in the cardiac mesoderm. Whereas, the phenotypes for Tup, Doc and *pnr* were consistent in all investigated mutants, the phenotype for Tin was mixed (i.e. reduction in and additional Tin-expressing cells were observed) in different *Iro-C* mutants (Figure 6 M-P and Figure 7 G, H). The phenotype in *Df(3L)iro-2* embryos that lack all three *Iro-C* members was a reduction in Tin-positive cells (Figure 6 M, N, T) at stage 11/12. The presence of additional Tin-positive cells was the predominant phenotype observed in *iroDFM1* embryos (Figure 6 M, O, T), while only some embryos exhibited reduced Tin expression. *Df(3L)iro-2/iroDFM1* embryos were characterized by a mild reduction of Tin-expressing cells (Figure 6 M, P, T) but some of these embryos also exhibited additional Tin-expressing cells. A plausible explanation for this incongruity of Tin phenotypes, which was observed in *Df(3L)iro-2* and *iroDFM1* embryos, is that in the *Iro-C* deficient mutant (*Df(3L)iro-2*) an additional gene (or genes) is (are) deleted, which might be also regulating Tin expression. Embryos that are mutant for *mirr* were characterized by overproduction of Tin-positive cells (Figure 6 T and Figure 7 G, H). During embryonic stage 11, Tin is detected in heart precursors (arrows in Figure 3 D) and in visceral mesodermal cells (asterisks in Figure 3 D) (Lockwood and Bodmer, 2002). Since the early expression of Ara/Caup encompasses the visceral mesoderm (Figure 3 C, D and Carrasco-Rando et al., 2011) a double labeling for Tin and a visceral mesodermal marker anti-FasciclinIII (Fas III) was performed. Tin and FasIII expression in
the visceral mesoderm was reduced to variable extents in all investigated mutants (Figure 6 M-P and Figure 7 G, H).

Figure 6. Lack of Iro-C affects the normal expression of early cardiac transcription factors *tup, Doc* and *tin* but not *pnr*.

*Tup, Doc2, pnr* and Tin expression was analyzed in different mutants for *Iro-C* and compared with their wildtype (WT) expression (A, E, I, M). The schematic illustration above each column represents the genetic background, where the presence of the box represents the presence of the indicated factor. (B-D) Embryos that are mutant for *Iro-C* are characterized by a reduction in Tup-expressing cells (arrows). (F-H) Lack of the entire *Iro-C* or *ara* and *caup* results in a dramatic reduction of Doc2 expression (arrows). Doc2 is also detected in the amnioserosa (asterisks in E and F). (J-L) Expression of *pnr* mRNA is unchanged in these mutants. Arrows in J-L point to missing Eve-positive clusters. (N) Tin expression is reduced in *Df(3L)iro-2* embryos (arrows) in both cardiac and visceral mesoderm. (O, P) The predominant phenotypes are shown for *iroDFM1* and *Df(3L)iro-2/iroDFM1* embryos. The presence of additional Tin-positive cells in the cardiac mesoderm (arrowheads in O) was observed in *iroDFM1* embryos and loss of some Tin-expressing cells (arrows in P) was the phenotype in *Df(3L)iro-2/iroDFM1* heterozygous embryos. (M-P) Tin and FasIII expression in visceral mesoderm was reduced in all *Iro-C* mutants tested. (Q-T) Quantification of *Tup* (Q), Doc2 (R), *pnr* (S) and Tin (T) phenotypes. All images are lateral views of 10/11 stage embryos; anterior is to the left.
Figure 7. Effect of lack of mirr on the expression of early cardiac transcription factors.
Tup, Doc2, pnr and Tin expression was analyzed in mirr<sup>e48</sup> embryos and compared with their wildtype expression (A, C, E, G). The schematic drawing above the figure depicts the genetic background of analyzed embryos. (A, B) Lack of mirr results in a reduction of Tup expression (arrows). (C, D) Doc2 expression was moderately reduced in mirr mutants (arrows). Asterisks in A, C and D point to Tup and Doc2 expression in the amnioserosa. (E, F) Similar to ara/caup mutants, pnr mRNA expression remains normal in mirr mutants. Arrow in F points to a missing Eve cluster. (H) mirr mutants exhibit an increased expression of Tin in cardiac mesoderm (arrowheads) and a reduced expression of FasIII and Tin in visceral mesoderm (arrow).

Taken together, these data indicate that Iro-C is required for early cardiogenesis, since the expression of the crucial cardiogenic factors, Tup, Doc and Tin, was affected in Iro-C mutants. Moreover, although pnr mRNA expression was unchanged in all investigated mutants, it was not sufficient to maintain the normal expression of tup, Doc and tin in cardiac mesoderm in the absence of Iro-C.
3.3 Ara/Caup and Mirr expression requires the presence of early cardiac transcription factors and depends on Dpp and Wg

To further investigate the functional relationship between Iro-C and early cardiogenic factors, the mesodermal expression of Ara/Caup and Mirr was analyzed in tup (tup^{1A-}), Doc (Df(3L)DocA), pnr (pnr^{VX6}) and tin (tin^{346}) mutant embryos (Figure 8). Results of these analyses revealed that Ara/Caup expression was downregulated in all tested cardiac mutants (Figure 8 A-F). Embryos mutant for tin and Doc were characterized by a dramatic loss of Ara/Caup expression (Figure 8 A, C, E, F). Although pnr mutants also exhibited a dramatic reduction in Ara/Caup expressing cells, remaining Ara/Caup expression was often detected in segmentally arranged “stripes” along the dorsal mesoderm (Figure 8 A, D). Mirr expression was only mildly reduced in tup mutants, in contrast to a strong reduction of Ara/Caup expression in this mutant (Figure 8 G, H and A, B). Embryos mutant for Doc, pnr and tin showed a strong reduction in, or lack of, Mirr-expressing cells (Figure 8 G, I-L). Ara/Caup and Mirr phenotypes in tup, Doc, pnr and tin mutants were quantified and are shown in Figure 8 M.

Thus, these results suggest that tup, Doc, pnr and tin are required for initiation and/or maintenance of Ara/Caup and Mirr in the mesoderm. Together with previous results showing that Iro-C is required for normal expression of Tup, Doc2 and Tin (see 3.2) in the cardiac mesoderm, these data demonstrate an interdependency of the members of Iro-C and the crucial cardiac transcription factors, which further supports the hypothesis that ara, caup and mirr function together with tup, Doc, pnr and tin and affect the proper generation of cardiac mesoderm.
Results

Figure 8. *tup, doc, pnr* and *tin* are required for the expression of Ara/Caup and Mirr in the mesoderm.

(A) Wildtype embryo expressing Ara/Caup and Eve in the mesoderm. (B) *tup* mutant embryos exhibit a strong reduction in Ara/Caup expression at the dorsal edge of the mesoderm (arrows). (C) Doc mutants exhibit a dramatic loss of Ara/Caup-positive expression in the dorsal mesoderm (arrows). (D) Lack of *pnr* limits the Ara/Caup expression to stripes-like patches (asterisks), located on the dorsal side of *pnr<sup>VX6</sup>* embryos. Arrows point to loss of Ara/Caup cells in the mesoderm. (E) Wildtype embryo expressing Ara/Caup and Dmef2. (F) *tin* mutants exhibit a reduction in Ara/Caup expression (arrows). (G) Wildtype embryo expressing Mirr and Eve. (H) Mirr expression is mildly reduced in *tup<sup>111</sup>* embryos (arrows). (I) Doc mutants show a dramatic loss of Mirr (arrows) in the dorsal mesoderm. (J) Mirr expression is severely reduced (arrows) in the embryos mutant for *pnr*. (K) Wildtype embryo expressing Mirr and Dmef2. (L) *tin* mutant embryos exhibit a strong reduction in Mirr-expressing cells (arrows). (M) Histogram showing quantification of Ara/Caup and Mirr phenotypes in *tup<sup>111</sup>, Df(3L)DocA, pnr<sup>VX6</sup> and *tin<sup>146</sup>* embryos. Lateral views of stage 10/11 embryos are shown.
It is well established that the two growth factors Dpp and Wg are very important for heart development (Azpiazu and Frasch, 1993; Bodmer, 1993; Lockwood and Bodmer, 2002; Park et al., 1996; Wu et al., 1995). Embryos lacking either of these secreted molecules do not form a heart (Frasch, 1995; Park et al., 1996; Wu et al., 1995; Yin and Frasch, 1998; Zaffran and Frasch, 2002). As the members of the Iro-C are expressed in both germ layers, in the ecto- and in the mesoderm, (Figure 4 and Calleja et al., 2000; Carrasco-Rando et al., 2011; Letizia et al., 2007), it is possible that the lack of Iro-C members might affect Dpp and Wg expression, which would result in an early cardiac phenotype. To address this point, the expression of dpp mRNA and Wg protein in the ectoderm was analyzed in Df(3L)iro-2 embryos. Dpp and Wg expression remained unchanged in Iro-C deficient mutant embryos (Figure 9 A-D), suggesting that the early cardiac phenotype observed in Iro-C mutants is not due to abnormal expression of Dpp or Wg in the ectoderm. Consistent with the previous findings, the expression of pnr mRNA in the ectodermal layer is independent of Iro-C (Figure 9 E, F), whereas ectodermal expression of Tup requires the presence of Iro-C (Figure 9 G, H).
Figure 9. Wg, dpp and pnr expression do not require Iro-C in the ectoderm in contrast to Tup. (A, C) Wildtype embryo stained for dpp mRNA (A) and for Wg protein (C). (B, D) Iro-C mutant embryos express dpp (B) and Wg (D) normally. (E, F) In Df(3L)iro-2 embryos, ectodermal pnr mRNA expression (F) is indistinguishable from wildtype (E). (G, H) Reduction of Tup-expressing cells is observed in the ectoderm in Iro-C deficient mutants (arrows in H) in comparison to the wildtype (G). Asterisks in G and H point to Tup staining in the amnioserosa. Lateral views of stage 10 embryos are shown.
It is known that Dpp signalling regulates Iro-C expression in the medial notum (Letizia et al., 2007). To determine whether Dpp signalling might affect Ara/Caup expression in the dorsal ectoderm and/or in the mesoderm, brinker (brk) was expressed in the ecto- and in the mesoderm. Brinker has been demonstrated to be a transcriptional repressor of Dpp target genes (Kirkpatrick et al., 2001; Saller and Bienz, 2001; Zhang et al., 2001). Overexpressing brk in the mesoderm (using the twi-Gal4; 24B-Gal4 mesodermal driver) resulted in a severe loss of Ara/Caup expression in the mesoderm (81%; n=37) (Figure 10 A, B). In contrast, Ara/Caup expression remained unaffected in the embryos that overexpress brk in the ectoderm (using the ectodermal driver 69B-Gal4) (Figure 10 C, D). These results suggest that Ara/Caup expression might be regulated differently by Dpp signalling in different germ layers.

Figure 10. Mesodermal overexpression of brk causes loss of Ara/Caup in the mesoderm, whereas overexpression of brk in the ectoderm does not affect Ara/Caup.
(A, C) Stage 10 wildtype expression of Ara/Caup and Eve in the mesoderm (A) and Ara/Caup and En in the ectoderm (C). (B) Ara/Caup expressing cells and Eve-positive cell clusters (arrows) are absent in the mesoderm of Drosophila embryos that overexpress brk in the mesoderm. (D) Ectodermal overexpression of brk did not have an effect on the expression of Ara/Caup in the ectoderm. Lateral views of stage 10/11 embryos are shown.

To determine the impact of Wg on Iro-C expression in the dorsal ectoderm, Ara/Caup expression was analyzed in the embryos that are mutant for wg (wg\textsuperscript{C\textsubscript{26}V}). In fact, Wg has been demonstrated to regulate the expression of Iro-C in the eye disc (Cavodeassi et al., 1999; Lee and Treisman, 2001; Maurel-Zaffran and Treisman, 2000). An immunostaining for Ara/Caup in wg mutant embryos revealed a dramatic loss of Ara/Caup in the ectoderm at stage 10/11 (Figure 11 A, B). The expression of Ara/Caup in the ectoderm was also reduced in dpp\textsuperscript{d6} and pnr\textsuperscript{V\textsubscript{N6}} embryos (Figure 11 A, D). These results...
clearly demonstrate a requirement for Wg signalling for the expression of Ara/Caup in the dorsal ectoderm and a requirement for Dpp signalling in both germ layers.

Figure 11. Requirement of wg, dpp and pnr for Ara/Caup expression in the dorsal ectoderm.
(A) Wildtype embryo double labelled for Ara/Caup and En. (B) Embryos mutant for wg are devoid of Ara/Caup expression (arrows). (C) dpp mutant embryos exhibit a reduction in Ara/Caup-expressing cells in the ectoderm (arrows). (D) pnr mutant embryos are characterized by a reduction of Ara/Caup cells in the ectoderm (arrows). Asterisks in C and D point to the Ara/Caup staining in the amnioserosa. Lateral views of stage 10/11 embryos.

To further explore the role of Dpp, pnr and tup in regulating Ara/Caup expression, the late expression of Ara/Caup was analyzed in embryos mutant for dpp, pnr and tup. At stage 15/16, the Ara/Caup phenotype in dpp<sup>d6</sup> and pnr<sup>VX6</sup> embryos was almost identical, exhibiting a pronounced reduction/loss of Ara/Caup-positive cells that are located along the heart tube, whereas the expression of Ara/Caup remained unchanged in the anteriorly located Eve-positive pericardial cells (Figure 12 A-C). As for tup mutants, Ara/Caup-positive cells were mostly present at this stage (Figure 12 A, D). Additionally, these mutants were characterized by a disorganization of Eve-positive pericardial cells.
3.4 The Iroquois complex is required for the formation of the Drosophila heart

It is evident that the Iro-C is required for the normal expression of Tup, Doc and Tin (see 3.2). This suggests a function for Iro-C in early events of cardiogenesis, such as the proper specification of mesodermal cells towards a cardiac fate. To determine how the differential effects on cardiac markers at early stages affects the formation of the dorsal vessel, heart phenotypes were analyzed in different Iro-C mutants at late stages of cardiogenesis.

A reduction of Odd-expressing pericardial cells was observed in Iro-C deficient (Df(3L)iro-2) mutant embryos at stage 16 (Figure 13 A, B). In contrast to Df(3L)iro-2, iro\textsuperscript{DFM1} and Df(3L)iro-2/iro\textsuperscript{DFM1} embryos were characterized by an increase in the number of Odd-positive pericardial cells that is statistically significant (Figure 13 A, C, D, Q). Of note, the quantification of heart phenotypes presented in Figure 13 Q-T includes also the heart phenotypes analyzed in mirr mutants shown in Figure 14. Similar to ara/caup mutants, mirr\textsuperscript{e48} embryos also had additional Odd-expressing pericardial cells (Figure 14 A, B and Figure 13 Q). All investigated mutants were characterized by an abnormal expression of Tin. Additional Tin-expressing cells were observed frequently (Figure 13 E-
H, R and Figure 14 C, D). Usually, Tin expression is detected in four out of six myocardial cells and in six pericardial cells in each hemisegment. However it is difficult to detect and distinguish some of the Tin-positive pericardial cells because they lie behind the myocardial cells (Jagla et al., 2002). An additional phenotype observed in \(i^{DFM1}\) and \(m^{e48}\) embryos was the detachment of Tin-expressing pericardial cells (Figure 13 E, G and Figure 15 A-C).

The presence of additional Odd-expressing pericardial cells and the fact that the number of Dmef2-positive myocardial cells was not increased in \(ara/caup\) and \(mirr\) mutants (see below) suggests that the additional Tin-positive cells belong to the pericardial cell type. To confirm this hypothesis, the expression of the extracellular matrix protein Prc was analyzed in \(i^{DFM1}\) and \(m^{e48}\) embryos. The Prc expression domain in \(ara/caup\) and \(mirr\) mutants is broader in comparison to its expression in wildtype embryos and Tin-positive cells are surrounded by Prc in these additional and detached Tin-positive cells (Figure 15 A, B, C). These findings indicate that \(ara/caup\) and \(mirr\) mutants are indeed characterized by the presence of additional Tin-positive pericardial cells.

To further verify defects in heart development caused by the lack of \(Iro-C\), the expression of the myocardial marker Dmef2 was analyzed. Embryos mutant for \(Iro-C (Df(3L)iro-2), ara\) and \(caup (i^{DFM1})\) and heterozygous embryos \((Df(3L)iro-2/i^{DFM1})\) were characterized by a mild reduction and misalignment of some Dmef2-positive myocardial cells (Figure 13 I-L). The Dmef2-expressing myocardial cells were counted in \(i^{DFM1}\) embryos and compared to those in wildtype \(Drosophila\) embryos. The results of the counting revealed that the number of Dmef2 cells was slightly reduced (statistically not significant) in \(ara/caup\) mutants, with an average number of Dmef2 myocardial cells of 101 \((n=22)\) compared to wildtype embryos (average number of Dmef2 myocardial cells is 104; \(n=22\)). As to the Dmef2 phenotype in \(m^{e48}\) embryos, only a mild reduction of Dmef2-expressing cells was observed but no misalignment of myocardial cells was detected in these embryos (Figure 14 E, F). However, the penetrance of the Dmef2 phenotype in \(mirr\) mutants was higher compared to other \(Iro-C\) mutants tested (Figure 13 S).
Figure 13. Heart phenotypes of Iro-C mutants.
The schematic drawing above each column illustrates the genetic background of the embryos that were used. (A-D, Q) An increase in Odd-expressing pericardial cells was observed in the embryos that lack ara and caup (arrowheads in C and D), whereas the embryos harbouring the larger chromosomal deletion (Df(3L)iro-2) exhibited a reduction in Odd-positive pericardial cells (arrows in B). Asterisks in A and D point to Odd expression in the gut. (Q) The difference in number of Odd-positive pericardial cells in each Iro-C mutant and in the wildtype embryos is statistically significant (Mann-Whitney test; * p < 0.01, ** p < 0.001, *** p < 0.0001). Of note, only Odd-positive PC were counted (Odd-positive lymph gland cells are not included). (E-H) Iro-C mutants show an abnormal Tin expression at stage 14-15. The brackets are placed to highlight the hemisegments in which Tin expression is disorganized. The arrowheads in G point to detached Tin-expressing cells. (I-L) Lack of Iro-C members resulted in a reduction of Dmef2 expressing myocardial cells (arrows) and misalignment of the two myocardial cell rows (arrowheads). (M-P) Iro-C mutants exhibit a loss of some Eve-positive clusters (arrows). (R-T) Histograms showing the quantification of the phenotypes. A-L are dorsal views of embryos at stage 14-16; M-P are lateral views of stage 10-11 embryos.
Eve-positive cells were also affected by lack of Iro-C genes at stage 10/11. A loss of some Eve-positive clusters was detected in all investigated mutants (Figure 13 M-P and Figure 14 G, H) with a more severe phenotype seen in the embryos deficient for Iro-C (Df(3L)iro-2) and in iroDFM1 mutant embryos (Figure 13 M-O). Moreover, the quantification of the Eve phenotype indicated that ara/caup appear to have more impact in regulating Eve expression in the mesoderm than mirr because only 15% of the analyzed mirr mutant embryos showed a phenotype for Eve (Figure 13 T). Also, the number of
missing Eve cell clusters in *mirr* mutant embryos varied between 1-2, whereas *ara/caup* mutants were missing 4-6 Eve clusters.

**Figure 15. Iro-C genes regulate pericardial cell number.**

(A) A stage 16 wildtype embryo double labelled for Tin and Prc, where Prc highlights the basal membrane of the myocardial cell rows and is expressed around pericardial cells. (B, C) In *ara/caup* (B) and *mirr* (C) mutants additional and detached Tin-positive cells are localized lateral to myocardial cells and surrounded by Prc. Arrows in B point to additional Tin- and Prc-expressing cells, arrowheads in B and C point to detached Tin- and Prc-positive pericardial cells. Dorsal views of stage 15/16 embryos.

To determine if the mesodermal expression of either *ara* or *caup* would rescue the heart phenotype observed in *Df(3L)iro-2 UAS-ara;Df(3L)iro-2 and UAS-caup;Df(3L)iro-2* fly lines were generated and crossed with *twi-Gal4;Df(3L)iro-2* flies to express either *ara* or *caup* in the embryos deficient for Iro-C in the mesoderm. Unfortunately, *Df(3L)iro-2* embryos in which UAS-*caup* was overexpressed in the mesoderm are not viable (own observation and as reported earlier Cavodeassi et al., 2001; Ikmi et al., 2008). Therefore the rescue experiments were done using embryos in which *ara* was expressed in the mesoderm of *Df(3L)iro-2* embryos. These embryos were characterized by a loss of Eve-positive clusters at stage 10/11 (84%, n=86; Figure 16 A, B) and disorganization of Tin-expressing cells (74%, n=167; Figure 16 C, D). The Eve and Tin phenotypes were similar to what was observed in either the Iro-C deficient embryos or in wildtype embryos in which *ara* was overexpressed in the mesoderm (see Figure 17 I, J). This data demonstrates that the expression of only *ara* in *Df(3L)iro-2* embryos in the mesoderm is not sufficient to rescue the heart phenotype that was observed in this mutant. It is tempting to speculate that *ara* and *caup* (as well as *mirr*) may act together in regulating the normal expression of Eve and Tin and possibly the co-expression of *ara* and *caup* or the co-expression of all three Iro-C factors would rescue the cardiac phenotype. Another explanation can be that due to the large chromosomal deletion of *Df(3L)iro-2* embryos, expressing only *ara* was not sufficient to rescue the heart defects. At present these are hypotheses that need to be further elucidated.
Fig. 16. Eve and Tin phenotype after expressing ara in the mesoderm of Iro-C deficient embryos.

(A) Wildtype Eve expression in stage 10/11 embryos. (B) twi::UAS-ara;Df(3L)iro-2 embryos are characterized by a severe reduction of Eve-positive cell clusters. (C) Wildtype Tin expression in stage 16 embryos. (D) twi::UAS-ara;Df(3L)iro-2 embryos exhibit a dramatic disorganization of Tin-positive cells. Arrows always point to a lack of expression; arrowheads point to the presence of additional cells. A and B are lateral views, C and D are dorsal views of embryos.

Having analyzed heart phenotypes caused by the lack of Iro-C, gain of function experiments were performed using the Gal4-UAS system. To this end, either ara, caup or mirr was overexpressed in the mesoderm and the effects on heart marker expression were analyzed. Since ara/caup mutants were characterized by a reduction of Eve-expressing cell clusters, an overproduction of Eve-positive cells was expected in the embryos that overexpress UAS-ara or UAS-caup. However, approximately 30% of the embryos, that overexpress ara (n=137) or caup (n=154) in the mesoderm, were characterized by a reduction of Eve-positive clusters (Fig. 17 A-C). It is noteworthy that additional 10% of these embryos lacked Eve-positive clusters completely (data not shown). When UAS-mirr was overexpressed throughout the mesoderm, a reduction in Eve-expressing clusters was observed in only 14% of analyzed embryos (n=43) (Fig. 17 A, D). Since the expression of Eve was only mildly affected in mirr mutants, these findings further support the hypothesis that mirr has a lower impact on the regulation of Eve expression in comparison to ara/caup. As to the pericardial marker Odd, a reduction (and sometimes a disorganization) of Odd-positive pericardial cells was observed after overexpressing either UAS-ara or UAS-caup or UAS-mirr (Fig. 17 E, G, H), which, in this case, is the opposite phenotype to what was observed in Iro-C mutants. Embryos mutant for ara/caup or mirr were characterized by the presence of additional Odd-expressing pericardial cells. A dramatic disorganization of Tin-positive cells was observed in the embryos that overexpress either ara, caup or mirr (Fig. 17 I-L).
Results

Figure 17. Ectopic expression of *ara*, *caup* or *mirr* in the mesoderm induces a cardiac phenotype.

(A-D) Lateral views of stage 10/11 embryos stained for Eve. (A) Wildtype expression of Eve. (B-D) Embryos that overexpress *ara* (B), *caup* (C) or *mirr* (D) show a reduction of Eve-positive clusters (arrows). Arrowhead in C point to the Eve cluster, containing additional cells. (E-H) Dorsal views of stage 16 embryos stained for Odd. (E) Wildtype expression of Odd. (F) *twi;24B::UAS-ara* embryos show a mild disorganization of Odd-positive pericardial cells. Arrow in F points to missing Odd-expressing cells, arrowhead points to additional Odd-positive cells. (G) *twi;24B::UAS-caup* embryos show a dramatic reduction of Odd-expressing pericardial cells (arrows). (H) *twi;24B::UAS-mirr* embryos exhibit a pronounced reduction of Odd pericardial cells (arrows) and lymph gland (lg) cells. (I-L) Dorsal views of stage 16 embryos stained for Tin. (I) Wildtype expression of Tin. (J) *twi;24B::UAS-ara* embryos exhibit additional Tin-expressing cells in the anterior part of the embryo (arrowhead). (K, L) *twi;24B::UAS-caup* (K) and *twi;24B::UAS-mirr* (L) are characterized by severe disorganization of Tin-expressing heart cells (arrowheads). Arrow in K points to a lack of Tin-positive cells.

In summary, these results suggest that the appropriate levels of *ara*, *caup* and *mirr* are required for the correct expression of cardiac markers and also for proper heart development.

3.5 Caupolican binds to the *hand* enhancer and regulates *hand*-luciferase activity

The *Drosophila* bHLH transcription factor *hand* is expressed in the dorsal mesoderm starting from stage 11 in the cells that will give rise to circular visceral muscles. At stage 12, its expression is initiated in the developing heart and slightly later, from stage 13 onwards, in the developing lymph glands. The expression of *hand* in the lymph glands and in the heart cells (myocardial and pericardial cells) maintains throughout embryogenesis (Kolsch and Paululat, 2002; Sellin et al., 2006). The cardiac expression of *hand* is controlled by a 513 bp enhancer, which is located in the third intron and recapitulates the endogenous expression pattern of *hand* (Han and Olson, 2005; Sellin et al., 2006; Tao et al., 2007).
The *hand* enhancer sequence was subjected to an *in silico* analysis (http://www.genomatix.de) in which a putative Caup binding site, 5’ACAATAACT3’, was identified and tested by electrophoretic mobility shift assay (EMSA). The EMSA was performed using only the homeodomain and flanking amino acids fused to a GST-tag (GST-CaupHD; Figure 18 A), since it was not possible to obtain the full-length Caup protein. Indeed, the Caup homeodomain efficiently binds to the predicted binding site present in the *hand* enhancer *in vitro* (Figure 18 B). To test whether binding of Caup to the *hand* enhancer has an effect on the transcription of *hand*, a luciferase reporter system was used. To this end a *hand*-luciferase (*hand*-luc) reporter was generated, in which luciferase activity is driven by the entire third intron that encompasses the *hand* enhancer. Remarkably, Caup can activate luciferase over 20 fold, whereas Ara activated less strongly (Figure 18 C, cream-colored bars), suggesting that Ara can activate the luciferase activity driven by the enhancer region of *hand*, however, less efficiently compared to Caup. In contrast to *ara* and *caup*, *mirr* had no effect on *hand*-luciferase activity (Figure 18 C, cream-colored bars). To exclude the possibility that the low induction of *hand*-luc activity is due to a reduced amount (or lack) of *ara* and *mirr* transcripts a semiquantitative RT-PCR was performed (Figure 18 D). The PCR was done using cDNAs that were made from the RNAs isolated from the lysates of transfected cells, and using a gene-specific forward primer and a pAc5.1C vector-specific reverse primer. The pAc5.1C vector-specific primer was designed for the V5 epitope tag sequence that is also present in the mRNA transcripts of the transfected *ara*, *caup* and *mirr* cDNA.

Recent reports showed that the transcriptional activity of chicken Irx2 as well as of Caup can be modulated by the MAPK pathway through phosphorylation of Irx2 and Caup proteins (Carrasco-Rando et al., 2011; Matsumoto et al., 2004). The phosphorylation of Caup switches Caup from transcriptional repressor to an activator of the muscle identity gene *slouch*, as tested in a luciferase reporter assay (Carrasco-Rando et al., 2011). It is known that the MAPK pathway is constitutively active in S2 cells (Carrasco-Rando et al., 2011) and therefore it was of interest to test whether MAPK phosphorylation of Iro-C proteins can influence the *hand*-luc activation. To test this, the cells were treated with the MAPK inhibitor PD098059. The inhibition of the MAPK pathway resulted in a slight increase of *hand*-luc activity compared to when the MAPK pathway was not inhibited (Figure 18 C, blue bars) and this difference was not statistically significant. Thus, these results indicate that the MAPK pathway does not play a significant role in the regulation of *hand* by Caup, Ara or Mirr.
Results

Figure 18. Caup binds to a predicted binding site present in the hand cardiac enhancer and activates hand-luciferase.

(A) The amino acid sequence that was used to generate GST-tagged CaupHD protein. The homeodomain of Caup is highlighted in blue. (B) Electrophoretic mobility shift assay was performed using GST-CaupHD protein and a radiolabeled probe corresponding to the predicted Caup-binding site on the hand enhancer. The arrow and the arrowhead point to GST-CaupHD/hand enhancer complex, where the protein (GST-CaupHD) is specifically bound to the double- (arrow) or single-stranded (arrowhead) DNA sequence of the predicted binding site. The asterisk marks unspecific bands. The non-specific competitive probe poly(dIdC) did not interfere with the binding of GST-CaupHD, whereas addition of unlabeled wildtype (WT) oligonucleotides inhibited the binding. (C) Effect of Caup, Ara and Mirr on hand-luc activity in the absence and presence of the MAPK inhibitor (PD098059). Caup and Ara activate hand-luciferase, whereas Mirr hardly increased hand-luciferase activity above the basal level. Statistical analyses were performed using the Mann-Whitney test , *p < 0.05 shows statistically significant differences in hand-luc activity in the cells transfected with either construct compared to the cells transfected with the empty vector. (D) Results from the semi-quantitative RT-PCR confirm the presence of ara, caup and mirr transcripts in the presence and absence of the MAPK inhibitor, β-tubulin is used as a loading control.

It is possible that Caup may induce hand expression in the dorsal mesoderm at stage 11, where both factors are expressed. To test this hypothesis, a double labeling for the Ara/Caup and GFP was performed in the hand-GFP reporter fly line embryos. Unfortunately, it was not possible to detect any co-localization of Ara/Caup and Hand in the mesoderm at this stage, suggesting that Caup may not induce the expression of hand in the mesoderm at stage 11. To further investigate the potential of Caup to activate the hand enhancer, caup was overexpressed in the mesoderm of the hand-GFP reporter fly line. Overexpression of UAS-caup in the mesoderm, using the twi-Gal4 driver fly line resulted in a disorganization of hand-GFP expressing heart cells at stage 16 (Figure 19 A, B) similar to the severe disorganization of Tin-positive cells observed in the embryos that overexpress caup throughout the mesoderm (Figure 17 I, K). This phenotype could be due to interfering with regulatory events during early cardiogenesis and may not be specific for Caup acting on the hand enhancer. Interestingly, mesodermal overexpression of caup
results in a loss of hand-GFP expression in the lymph glands, whereas Odd expression was still present in the lymph glands (Figure 17 G).

Figure 19. Overexpression of caup causes a disorganization and reduction of hand-expressing cells
(A) Wildtype hand-GFP expression. (B) Embryos that overexpress caup in the mesoderm exhibit an overall disorganized heart as well as reduction of hand-expressing heart (arrow) and lymph gland cells (lg) cells.

Taken together the results from the EMSA and cell culture experiments show that Caup binds to the predicted binding site in the regulatory region of hand and activates hand-luc in vitro.
4 Discussion

4.1 *Iro-C* homeobox transcription factors are new components in the cardiac gene regulatory network

The results of this study describe the requirement of the *Drosophila Iro-C* for heart development. Their expression at embryonic stage 10/11 ensures the correct patterning of the dorsal mesoderm and subsequent development of the fly heart. Tissue patterning requires the precise coordination of instructive signals that are interpreted by a complex network of regulatory genes. The mesoderm of *Drosophila* is patterned along the anterior-posterior and dorso-ventral axis, possessing within each segment different domains that will be specified as cardiac, somatic and visceral mesoderm. In early embryonic stages, the coordinated action of Dpp and Wg specifies the *tin*-positive mesoderm as cardiac and somatic mesoderm (Lockwood and Bodmer, 2002; Park et al., 1996; Wu et al., 1995). Subsequently, the MAPK signalling pathway that is activated by FGFR and EGFR and the Notch pathway act together to further subdivide the cardiac and somatic mesodermal domains (Carmena et al., 2002; Grigorian et al., 2011; Halfon et al., 2000). The early expression pattern of *ara*, *caup* and *mirr* in combination with phenotypic analyses of *Iro-C* mutants suggest that the *Drosophila Iro-C* could affect dorsal mesoderm patterning, consistent with the fact that the members of the *Iro-C* are known to control patterning in different tissues.

It appears that the *Iro-C* factors contribute to the specification of the dorsal mesoderm derivatives in response to the above mentioned signalling pathways. In fact, such a regulation has been described in the wing imaginal disc, where EGFR and Dpp signalling regulates the expression of *ara* and *caup* (Letizia et al., 2007) which specify and pattern the territory of the developing notum by interacting with other transcription factors (de Navascues and Modolell, 2007; Wang et al., 2000; Zecca and Struhl, 2002). The third member of the *Iro-C*, *mirr*, also has been shown to act downstream of EGFR in eggshell patterning and dorso-ventral axis formation (Andreu et al., 2012; Fuchs et al., 2012). In addition, the *Drosophila Iro-C* genes are required for the specification and patterning of the eye, where *ara*, *caup* and *mirr* expression is regulated by Hh and Wg in the eye/antenna imaginal disc (Cavodeassi et al., 1999; Cavodeassi et al., 2000). As shown previously, *mirr* can be regulated by Unpaired (Upd), a ligand that can activate JAK/Stat
Janus kinase/signal transducers and activators of transcription) signalling during eye development (Gutierrez-Avino et al., 2009; Zeidler et al., 1999). The JAK/Stat pathway is active in the dorsal mesoderm and recently has been shown to be involved in dorsal vessel development (Johnson et al., 2011). Among other genes that might be regulated by the JAK/Stat signalling pathway, caup has been identified as a target of the transcriptional effector of the JAK/Stat pathway, Stat92E, in a chromatin immunoprecipitation assay (Johnson et al., 2011; Sotillos et al., 2010). Interestingly, the increase of Tin and Odd pericardial cells, observed in ara/caup and mirr mutants is reminiscent to what was observed in Stat92E \textsuperscript{M2} embryos (Johnson et al., 2011). Furthermore, similar to Stat92E\textsuperscript{M2} embryos, ara/caup mutants were characterized by a misalignment of myocardial cells. These findings lead to the hypothesis that in the dorsal mesoderm JAK/Stat signalling might regulate caup and possibly ara and mirr expression. To test this hypothesis, it will be necessary to express caup (as well as ara and mirr) in the mesoderm of stat mutants and determine whether it can rescue the mutant phenotype. Also, to determine to which extent the phenotypes of embryos mutant for ara/caup and for stat are similar, it will be interesting to analyze the phenotype for the crucial cardiac transcription factors, such as tup, Doc and pnr, in Stat92E embryos and to compare it to what was observed in iro\textsuperscript{DFM1} embryos at early stages of cardiogenesis. Taken together, this data suggests that the Drosophila Iro-C could act downstream of the above mentioned signalling pathways and control the proper development of the fly heart. Furthermore, the Drosophila Iro-C factors have been shown to regulate the expression of fringe, a glycosyltransferase that regulates Notch signalling in the developing eye and during oogenesis (Cavodeassi et al., 1999; Dominguez and de Celis, 1998; Zeidler et al., 1999; Zhao et al., 2000). One of the important functions of the Notch pathway in the dorsal mesoderm is the establishment of the proper number of heart and muscle progenitors (Carmena et al., 2002; Park et al., 1998). Knowing that Iro-C can regulate Notch activity, one might speculate that the absence of Iro-C may affect the correct development of myo- and pericardial cells and therefore results in an abnormal number of heart cells. At this point, this is a hypothesis and further experiments will be required to determine the molecular mechanisms of Iro-C function during cardiogenesis.

Since ara/caup and mirr are expressed not only in the mesoderm but also in the ectoderm it could be that the heart phenotypes in Iro-C mutants are due to ectodermal requirement of Iro-C genes. The fact that ectodermal Dpp and Wg expression was normal in the embryos deficient for Iro-C excludes the possibility that the defects in heart
development observed in *ara/caup* and *mirr* mutants are due to reduced levels of Dpp and/or Wg expression. However, to determine the germ layer specific contribution of *ara*, *caup* and *mirr* in cardiogenesis it is necessary to inhibit the function of each *Iro-C* member individually and tissue specifically. To this end, transgenic UAS-*ara*RNAi, UAS-*caup*RNAi and UAS-*mirr*RNAi fly lines were used. For this study, either UAS-*ara*RNAi or UAS-*caup*RNAi or UAS-*mirr*RNAi was expressed in the mesoderm and in the ectoderm. However, expressing either of the above mentioned constructs did not result in a heart phenotype. To test the efficiency of the RNAi lines the *daughterless*-Gal4 (*da-Gal4*) fly line was used to express either RNAi construct throughout the embryo, that should efficiently knock down either *ara*, *caup* or *mirr* in the entire embryo. Unfortunately, the results of the PCR showed that *ara*, *caup* and *mirr* transcripts were still present, i.e. it was not possible to inhibit either member of the *Iro-C* by use of these RNAi lines.

To determine whether *Iro-C* could be integrated into the cardiac gene regulatory network in *Drosophila*, the interdependency between the core cardiogenic factors *tup*, *Doc*, *pnr* and *tin* and *Iro-C* was investigated during cardiogenesis and the results are summarized in Figure 20. Analyses of Ara/Caup and Mirr expression in *tup*, *Doc*, *pnr* and *tin* mutants demonstrated the requirement of all four factors for Ara/Caup expression. Pronounced loss of *ara/caup* and *mirr* expression in *tin* and *Doc* mutants shows that these factors act upstream of *Iro-C*. It has been reported previously that also the vertebrate homolog of *tin*, Nkx2.5, regulates the expression of Irx4 in the embryonic heart (Bruneau et al., 2000). Ara/Caup expression was also strongly downregulated in *tup*\textsuperscript{isl1} and in *pnr*\textsuperscript{y26} mutant embryos, however remaining expression of Ara/Caup was still present in these mutants, suggesting that *tup* and *pnr* also regulate the proper expression of *ara/caup* in the mesoderm.

Complementary experiments demonstrated that the *Drosophila* *Iro-C* is required for Doc expression in cardiac mesoderm and also to restrict the expression of Tin, since additional Tin-positive cells were the characteristic phenotype for *ara/caup* and *mirr* mutants. The phenotypic analyses of Tup expression revealed that it was only mildly affected in 19-27% of the analyzed embryos mutant for *ara/caup* and *mirr*. The expression of *pnr* was hardly affected in any of the investigated mutants for *Iro-C*, which was consistent with a previous report (Herranz and Morata, 2001). This fact is intriguing because *pnr* has been demonstrated to be required for the maintenance of *tup* and *doc* in the heart forming region (Mann et al., 2009; Reim and Frasch, 2005). However, *Iro-C* mutants were characterized by a reduction of Doc expression, although *pnr* was present.
One possible model can be that *pnr* requires the presence of *Iro-C* to function properly and to maintain Doc expression. The expression of *ara/caup* depends on *pnr* not only at early stages but also at stage 15/16. *Drosophila* embryos mutant for *pnr* were devoid of the seven pairs of Ara/Caup-expressing cells that are segmentally arranged along the heart at stage 15/16. A similar phenotype was observed in *dpp* mutants. Since *pnr* is a target of Dpp signalling in different tissues (Klinedinst and Bodmer, 2003; Sato and Saigo, 2000), this finding suggests that *ara/caup* could be regulated by Dpp through *pnr*. It is known that Pnr may regulate *Iro-C* expression in the wing and eye discs (Cavodeassi et al., 2000; Letizia et al., 2007; Maurel-Zaffran and Treisman, 2000). Additionally, the enhancer region of *ara/caup*, IroRE\(^2\), contains putative Pnr-binding sites and in the wing disc Pnr has been shown to activate the expression of the IroRE\(^2\)-lacZ construct (Letizia et al., 2007), which suggests that *pnr* could regulate the expression of *Iro-C* also in the mesoderm. Nevertheless, this does not rule out a possible direct regulation of *ara/caup* by Dpp in the mesoderm. In fact, loss of Ara/Caup was observed after mesodermal overexpression of *brk*. The transcriptional repressor *brk* is known to bind to *dpp*-response elements of some downstream targets of the Dpp pathway in the absence of Dpp signalling (Kirkpatrick et al., 2001). When UAS-*brk* was overexpressed in the ectoderm, using the 69B-Gal4 driver, Ara/Caup expression was normal in these embryos at stage 10/11. In contrast, *dpp* mutant embryos were characterized by a reduction of Ara/Caup expressing cells in the ectoderm. This suggests that *ara/caup* expression might be regulated differently by Dpp in the mesoderm and in the ectoderm. Since the 69B-Gal4 driver has been reported as a later onset ectodermal driver (Klinedinst and Bodmer, 2003), it is possible that *brk* was not expressed at sufficient high concentrations in these embryos at the stage when they were analyzed. Therefore, *ara/caup* expression remained unchanged in these embryos. Thus, the ectodermal expression of *ara/caup* may still be regulated directly by Dpp, however further experiments will be required to test this.
number of Tin-expressing cells and for normal expression of Doc in the cardiac mesoderm. Black arrows indicate previously described interactions; orange arrows indicate novel interactions with Iro-C, as proposed in the present work.

Taken together, this data place the Iro-C into the gene network regulating heart development (Figure 20). Moreover, these findings challenge our current understanding of interactions between the well characterized transcription factors and will spur additional investigations of this subject.

### 4.2 Identification of novel heart-associated cells and a new pericardial cell type

Detailed analyses of the expression pattern of Ara/Caup and Mirr identified a new population of heart-associated cells. At late embryonic stages, Ara/Caup and Mirr are detected in seven pairs of Ara/Caup/Mirr-positive cells and seven pairs of Mirr only expressing cells located laterally to the dorsal vessel. Since none of the known pericardial cell markers co-localizes with Ara/Caup- or Mirr-positive cells, it led to the conclusion that these cells may represent newly identified heart-associated cells. The seven pairs of Ara/Caup/Mirr- and Mirr- only expressing cells were initially thought of being connected with the seven pairs of alary muscles that attach the dorsal vessel to the dorsal epidermis (LaBeau et al., 2009). However, a double immunostaining for Mirr and Prc, which is also expressed along the seven pairs of alary muscles demonstrated that Mirr-positive cells are rather located between the Prc-positive extensions of the alary muscles (Chartier et al., 2002). Data presented in the current work identifies two subtypes of Ara/Caup-positive cells: the heart associated cells and a novel subtype of pericardial cells that co-express Eve
and Ara/Caup in the anteriorly located eight Eve-expressing pericardial cells. Prior to this study, five pericardial cell types were known: Eve pericardial cells, Tin pericardial cells, Tin and Lb pericardial cells, Odd pericardial cells and Odd and Svp pericardial cells (see Figure 2) (Azpiazu and Frasch, 1993; Bodmer, 1993; Frasch et al., 1987; Jagla et al., 1997; Ward and Skeath, 2000). This interesting finding suggests yet another role for Ara/Caup in the process of cardiogenesis, a role in the diversification of pericardial cell types. Furthermore, the identification of Ara/Caup- and Mirr-expressing cells increases the complexity of the cell types associated with the Drosophila heart. In the future, it will be interesting to determine the developmental fate and the functional significance of these cells.

4.3 Hand is a direct target of Caup in vitro

Iro-C transcription factors are known to regulate different developmental processes, however little is known about their direct targets. Previous studies showed that the Drosophila Iro-C may directly regulate the expression of fringe, encoding the glycosyltransferase that regulates Notch activity in different tissues and slouch, a muscle identity gene in the somatic muscle cells (Carrasco-Rando et al., 2011; Cavodeassi et al., 1999; Dominguez and de Celis, 1998; Zeidler et al., 1999). Here, a previously unknown binding site for Caup is presented. The binding site has been identified in the enhancer region of hand that contains the regulatory sequences necessary to recapitulate hand expression in the visceral mesoderm and in myocardial and pericardial cells (Sellin et al., 2006). Since hand starts to be expressed at late stage 11 in the dorsal mesodermal domains that give rise to the circular visceral musculature (Kolsch and Paululat, 2002), it was hypothesized that Caup might regulate hand expression and thereby pattern the dorsal mesoderm. Indeed, Caup does bind to the hand enhancer and Caup is a very potent activator of a hand-luciferase construct in vitro, however, it was not possible to show a co-expression of Caup and hand at stage 11 in the visceral mesoderm. Kölsch and Paululat (2002) showed that Hand is expressed in Eve pericardial cells by stage 12/13 and a coexpression of Ara/Caup and Eve in a subset of Eve pericardial cells was demonstrated in the current work. It is thus plausible that Caup regulates hand expression in these cells. In vertebrates it was shown that the ventricular expression of Hand1 is regulated by Irx4 (Bruneau et al., 2001). It is also possible that Caup regulates hand expression in a not yet
identified location where both factors are co-expressed. Mesodermal overexpression of UAS-caup did not result in an overproduction of hand-expressing cells but rather in a reduction of hand expression in lymph gland cells and an overall disorganized heart, which was not surprising because severe disorganization of heart cells, in particular tin-expressing cells, was the characteristic phenotype observed after mesodermal overexpression of caup. Data presented in this study also indicates that the hand-luciferase activation by Caup is independent of MAPK signalling, in contrast to slouch activation by Caup, where the transcriptional activity of Caup was modulated by MAPK (Carrasco-Rando et al., 2011). To summarize regulatory aspects of Caup function a novel binding site for Caup has been identified and verified in vitro and the transcriptional regulation of Caup may differ in different contexts.
5 Summary

The heart of the fruit fly *Drosophila* is a valuable model for studying regulatory genes that are involved in cardiogenesis. The fly heart has a simple linear structure and consists of contractile myocardial cells and non-contractile pericardial cells. The *Drosophila* heart cells are specified in the dorsal mesoderm as a result of the coordinated action of Decapentaplegic (Dpp, TGFβ family) and Wingless (Wg, Wnt family) signaling and the cardiac transcription factors *tinman* (*tin*; Nk-2 factor), *pannier* (*pnr*; GATA factor), *Dorsocross* (*Doc*; Tbox factor) and *tailup* (*tup*; LIM-homeodomain factor).

The *Iroquois* gene complex (*Iro-C*) in *Drosophila* consists of the three homeobox transcription factors *araucan* (*ara*), *caupolican* (*caup*) and *mirror* (*mirr*). Members of the *Iro-C* participate in various developmental processes such as patterning and specification of different tissues in *Drosophila*. However, until now the role of the *Iro-C* in *Drosophila* heart development had not been analyzed.

To test the hypothesis that the *Drosophila* *Iroquois* transcription factors function in the cardiac gene regulatory network, the expression pattern of the *Iro-C* factors was characterized and heart phenotypes in different *Iro-C* mutant embryos were analyzed. Together, these experiments revealed a role for *Iro-C* in cardiogenesis. During early stages of heart development, *ara*, *caup* and *mirr* are expressed in the dorsal mesoderm suggesting that the *Iro-C* could be involved in patterning of the dorsal mesoderm. The characterization of the *Iro-C* expression pattern at late embryonic stages revealed the existence of a subpopulation of pericardial cells, which co-express Even-skipped (Eve) and Ara/Caup. This observation suggests that a greater diversity of pericardial cell types exists than was previously known. Additionally, seven pairs of Ara/Caup/Mirr-expressing and seven pairs of Mirr only expressing cells were identified along the heart tube, which represent a novel group of heart-associated cells.

Embryos mutant for *ara/caup* are characterized by a reduced expression of *Doc* and overproduction of Tin-positive cells in the cardiac region. The third member of the *Iro-C*, *mirr*, has only a minor effect on *Doc* expression compared to *ara/caup*, but overproduction of Tin-expressing cells in *mirr* mutants is observed as well. Embryos mutant for *ara/caup* or *mirr* exhibited an expansion of pericardial cells, particularly with respect to Tin, Odd-skipped (Odd) and Pericardin (Pre) expression at late embryonic stages. Thus, the
presented data demonstrates a requirement for ara, caup and mirr in heart development and that the Iro-C factors may regulate the number of pericardial cells.

To determine whether the members of the Iro-C might be integrated into the early cardiac gene regulatory network, the interdependency between crucial cardiogenic factors and Iro-C was investigated during heart development. Phenotypic analyses revealed that mesodermal Ara/Caup and Mirr expression depends on tin, pnr, Doc, tup as well as on Dpp signaling. The correct expression of Doc and tin requires the presence of ara/caup, demonstrating interdependency between these factors. The expression of tup was only mildly affected in some of the analyzed ara/caup or mirr mutants. Interestingly, pnr, whose expression is required to maintain Doc and tup in the cardiac mesoderm was not affected by lack of ara/caup, while Doc and tup expressing cells were reduced in these mutants. The fact that pnr is present but Doc and tup expression is still reduced suggests that pnr and Iro-C cooperate in maintaining Doc and tup expression. This finding is intriguing since it challenges our current understanding of interactions between cardiac transcription factors.

This study also shows that Caup binds to the enhancer region of hand, a bHLH transcription factor expressed in the visceral mesoderm and in the heart and that Caup regulates the transcriptional activity of the hand reporter construct in vitro.

Taken together, the results of this study identify the Iro-C as a new component in the gene transcriptional network that acts in the dorsal mesoderm and affects heart development in Drosophila. Moreover, the current work provides a new basis for re-investigating our current understanding of the complex interactions between the known cardiac transcription factors.
6 References


References


7 Curriculum Vitae

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Oral presentation 2011 Spring meeting of the International Graduate School in Molecular Medicine. Ulm, Germany
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PUBLICATION

8 Declaration

I hereby declare that I wrote the present dissertation with the topic: “Identification of a role for the Iroquois complex in Drosophila heart development” independently and used no other aids than those cited. In each individual case, I have clearly identified the source of the passages that are taken word for word or paraphrased from other works.

I also hereby declare that I have carried out my scientific work for the degree of Doctor of Philosophy in English under the guidance and supervision of Professor Dr. Michael Kühl in the research group of PD Dr. Petra Pandur, Institute of Biochemistry and Molecular biology, University of Ulm, Germany. The work was done according to the principles of good scientific practice in accordance with the current “Satzung der Universität Ulm zur Sicherung guter wissenschaftlicher Praxis” (Rules of the University of Ulm for Assuring Good Scientific Practice).

Place: Ulm, Germany Zhasmine Mirzoyan

Date: 29.07.2013 Signature
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