Heart Enhancers: Development and Disease Control at a Distance

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Bound by lineage-determining transcription factors and signaling effectors, enhancers play essential roles in controlling spatiotemporal gene expression profiles during development, homeostasis and disease. Recent synergistic advances in functional genomic technologies, combined with the developmental biology toolbox, have resulted in unprecedented genome-wide annotation of heart enhancers and their target genes. Starting with early studies of vertebrate heart enhancers and ending with state-of-the-art genome-wide enhancer discovery and testing, we will review how studying heart enhancers in metazoan species has helped inform our understanding of cardiac development and disease.

Keywords: gene regulation, cardiac gene expression, transcription factor (TF), epigenomics and epigenetics, comparative genomics, enhancer

INTRODUCTION

The heart is a vital organ whose primary role is to pump blood through the circulatory system to reach different organs. Heart-like structures are ancient and observed across diverse metazoans, including arthropods (such as Drosophila), mollusks (such as octopus) and chordates. Heart structures vary widely across metazoans ranging from a single-layered tubular heart in arthropods and tunicates (including Ciona), three separate hearts in some cephalopods (including octopus), a two-chambered heart in jawed fish, a three-chambered heart in amphibians, to a four-chambered heart in other tetrapods (reviewed in Stephenson et al., 2017; Poelmann and Gittenberger-de Groot, 2019). This lineage-specific tuning of cardiac structures is accompanied by changes in the whole circulatory system and highly adapted to the specific physiological needs of different animals. Despite these differences in heart structure, which are mostly related to later-stage heart morphogenesis, many cellular events and molecular regulators involved in early heart development are broadly shared across metazoan species.

A core set of cardiac transcription factors (TFs), including NK2 (Drosophila homolog: Tinman), MEF2 (Drosophila homolog: Me2), GATA (Drosophila homolog: Pannier), TBX (Drosophila homolog: Nmr1/2, Doc1/2/3, etc.), and HAND (Drosophila homolog: Hand) families, interact with enhancers to control cardiac gene expression and cell fates in Drosophila, fish, and tetrapods (reviewed in Olson, 2006; Tolkin and Christiaen, 2012; Waardenberg et al., 2014). Though specific usage of paralogs and dosage sensitivities may vary between different species, these core TFs form the “cardiac regulatory kernel” (Tolkin and Christiaen, 2012; Waardenberg et al., 2014) in metazoans by closely interacting with each other and extracellular signaling cues. The requirement of extracellular signaling pathways in cardiogenesis also shows a high degree of conservation. The
core signaling pathways, such as WNT, FGF, NOTCH, and BMP, play essential cardiogenic roles in both Drosophila and vertebrates (reviewed in Noseda et al., 2011). Early vertebrate heart development involves a conserved sequence of cellular events that are seen in most, if not all, classes of vertebrate species (reviewed in Miquerol and Kelly, 2013). These events include: the emergence of specified cardiac progenitors within the anterior lateral plate mesoderm; migration of the cardiac progenitors to the midline to form the linear heart tube; rightward looping and elongation of the primitive heart tube; ballooning of the atrial and ventricular chambers out from the looped tube; and cardiac cushion and valve formation at the atrioventricular canal and outflow tract. This conserved set of events involve the complex interplay of multiple cardiac cell types, including the first heart field progenitors (FHF) that give rise to the linear heart tube and second heart field progenitors (SHE) that provide later addition to both poles of the heart tube (Kelly, 2012). Although cardiomyocytes make up a significant portion of mature hearts, other cell types, such as endocardial cells, smooth muscle cells, and cardiac fibroblasts, are also involved in cardiac development and physiological function (Hu et al., 2018; Honkoop et al., 2019; Tucker et al., 2020).

Understanding the interplay between multiple cardiac TFs and signaling pathways, within and between the cell types involved in cardiogenesis, requires a detailed knowledge of the cis-regulatory elements (CREs) that comprise heart enhancers. The regulatory logic encoded within CREs is readily understood by the embryo and is sufficient to organize multiple cardiac TFs and signaling pathways that ultimately result in a fully formed and functioning heart. In contrast, it has taken decades of experimental advances and insights to develop systems and technologies where cardiac CREs can be discovered and tested.

In this review, we discuss the genetic control of heart development and disease from an enhancer-centric perspective. From early gene-centric enhancer dissection in the 1990s to genome-wide characterization of heart enhancers in development and disease today, the discovery of heart enhancers has substantially shaped our understanding of the principles in cardiac gene regulation. We begin with a brief overview of developmental enhancers followed by a discussion of regulatory principles gained from pre-genomics enhancer studies. We then discuss how rapid advances in genome-wide approaches have transformed our knowledge regarding the locations, interactions, temporal dynamics and functions of heart enhancers. Our review will incorporate evolutionary characteristics of heart enhancers and discuss how new methods for dissecting heart enhancer functions promises to improve our understanding of heart development and cardiovascular diseases.

ENHANCER STRUCTURE AND FUNCTION IN DEVELOPMENT: A PRIMER

Enhancers are traditionally defined as short non-coding DNA sequences with the ability to drive gene expression regardless of the genomic distance, position, and orientation relative to the cognate genes [i.e., (Blackwood and Kadonaga, 1998) recently reviewed by Field and Adelman, 2020]. Enhancers can influence gene expression over short (hundreds of base pairs, bp) or large (megabases) genomic distances. These distal enhancers form long-range chromatin interactions with their target genes, such as the well-studied ZRS enhancer that is 1 Mb away from its target Shh (Lettice et al., 2003). This flexibility allows a single gene to be regulated by multiple enhancers with different spatiotemporal activities, as well as a single enhancer to contribute to the regulation of multiple genes, which was shown in recent genome-wide enhancer interaction maps (Montefori et al., 2018; Jung et al., 2019). Together this many-to-many relationship sets up a complex regulatory network to achieve the highly diverse tissue-specific expression patterns evident in development.

Spatial-temporal developmental gene expression is achieved through the combinatorial recruitment of a discrete set of TFs to enhancers (for a recent review of how TFs recognize CREs see Zeitlinger, 2020). TFs interact with enhancers through short degenerate DNA sequence motifs. Recent work investigating the regulatory logic of a typical developmental enhancer supports an overarching principle that specific developmental gene expression relies on sub-maximal TF recognition motifs (Farley et al., 2015). Layered on top of TF motif affinity is the motif syntax within an enhancer, where the spacing, orientation, and order of the motifs themselves can impact the ability of the enhancer to drive developmental gene expression (Farley et al., 2016). It is also important to recognize that developmental genes are commonly regulated by additional redundant enhancers and ascertaining the contributions of individual enhancers remains an outstanding challenge for the majority of developmentally expressed genes (Cannavò et al., 2016; Osterwalder et al., 2018).

Some lineage-determining TFs can bind to compact chromatin regions that are largely inaccessible to other factors. These pioneer factors recruit chromatin-remodeling complexes that promote nucleosome eviction, facilitating the subsequent binding of other collaborating TFs and signal effectors (McPherson et al., 1993; Cirillo et al., 2002; reviewed in Zaret, 2020). To impact gene expression, TFs recruit transcriptional cofactors to enhancers. Cofactors can in turn modify chromatin states by catalyzing post-translational histone modifications (e.g., P300/CBP MLL3/4), initiate chromatin remodeling (e.g., BRG1), bridge the gap between promoters and enhancer-bound transcription machinery (e.g., Mediator), or affect the affinity of TF binding at enhancers (Malik and Roeder, 2010; Siggers et al., 2011; Slattery et al., 2011; Krasnov et al., 2016). Despite these advances (and many others), much remains to be learned about the mechanisms underlying the recruitment of pioneer factors to a small subset of genomic sites and the molecular events that follow.

Enhancer activation in development is accompanied by progressive changes at the chromatin level, which in turn can be used to annotate enhancer states. Repressed enhancers are located in nucleosome dense regions. Certain repressed regions are characterized by the post-translational histone modification H3K27me3 which is deposited by the
Polycomb repressive complex 2 (PRC2). The binding of pioneer factors and chromatin-remodeling complexes may switch enhancers to a poised state, in which enhancers share many features with those in an active state. Poised enhancers show features of low nucleosome occupancy, limited TF binding, and post-translational histone modifications H3K4me1 and H3K4me2 without the presence of H3K27ac, a histone mark of active developmental enhancers (Creyghton et al., 2010; Rada-Iglesias et al., 2011; Zentner et al., 2011). These poised developmental enhancers may even retain the repressive mark H3K27me3 (Rada-Iglesias et al., 2011; Zentner et al., 2011). Upon full activation, transcription co-factor P300 and RNA polymerase II are recruited to enhancers, leading to bi-directional transcription of enhancer RNAs and active enhancer regions marked with H3K27ac (reviewed by Calo and Wysocka, 2013; Heinz et al., 2015).

Enhancer activities are influenced by both local chromatin interactions and higher-order chromatin architectures. Eukaryotic genomes are compartmentalized into large self-interacting chromatin domains, termed topologically associated domains (TADs) (Dixon et al., 2012; Rao et al., 2014). TADs

![Image of cardiac TF-enhancer interactions](FIGURE 1 | Early examples of validated cardiac TF-enhancer interactions. The first exons of the cardiac genes are shown in dark blue. Enhancer elements are shown as gray boxes. The AR1 enhancer of mouse Nkx2.5 contains a repressive element in the middle, which is shown in black. Direct activators are listed above the enhancer elements while repressors are shown below. Upstream factors without direct binding evidence are indicated with dotted lines. E1: exon 1. These schematics are generated based on data from these publications: mouse Nkx2.5 (Searcy et al., 1998; Lien et al., 1999, 2002; Liberatore et al., 2002; Brown et al., 2004; Chi et al., 2005; Takeuchi et al., 2005; Chen and Cao, 2009; Clark et al., 2013; Doppler et al., 2014; Quinodoz et al., 2018); Chicken NKX2.5 (Lee et al., 2004); Mouse Gata4 (Rojas et al., 2005; Schachterle et al., 2012); zebrafish gata4 (Heicklen-Klein and Evans, 2004); mouse Gata6 (Molkentin et al., 2000); Chicken GATA6 (He and Burch, 1997; Davis et al., 2001; Adamo et al., 2004); mouse Mef2c (Dodou et al., 2004; Takeuchi et al., 2005; Pane et al., 2018); mouse Hand2 (McFadden et al., 2003).)
largely constrain the chromatin span that enhancers search through and define the regulatory domains within which enhancer-promoter interactions most frequently occur (Long et al., 2016). For example, promoter capture Hi-C experiments have revealed that 60–80% of the detected promoter interactions occur within TADs (Javierre et al., 2016; Choy et al., 2018; Montefiori et al., 2018). Early studies have noticed that TAD boundaries are shared between different cell types and conserved between species (Dixon et al., 2012; Vietri Rudan et al., 2015), however, these two concepts have been revised more recently. An increasing number of studies reported dynamic loss and gain of TADs and changes of TAD sizes during differentiation (Bonev et al., 2017; Bertero et al., 2019; Zhang et al., 2019). While evolutionarily conserved TADs correspond to regions of conserved synteny harboring important developmental genes and enhancers (Harmston et al., 2017), new analyses have questioned the extent to which TAD boundaries themselves correspond to evolutionary breakpoints (Eres et al., 2019; Eres and Gilad, 2020; Torosin et al., 2020). The importance of understanding how TADs relate to gene regulation is underscored by the increasing number of experiments showing that the disruption of TAD boundaries and sub-TAD domains can rewire enhancer-promoter interactions and fundamentally change the regulatory environment (Guo et al., 2015; Lupiáñez et al., 2015; Flavahan et al., 2016; Franke et al., 2016; Liang et al., 2020).

In sum, the precise and robust transcriptional regulation that occurs during development is achieved by the complex interplay between enhancers, TFs, co-factors, and epigenetic modifications, which together are organized under higher orders of chromatin architectures.

**HEART ENHANCERS: FUNDAMENTAL INSIGHTS, ONE CRE AT A TIME**

Studies of heart enhancers initiated from targeted searches around cardiac genes. Putative enhancer regions were screened by “promoter shattering” in which regulatory regions near the TSS are narrowed down via a series of deletions/mutations to produce overlapping DNA segments that are tested in reporter assays (Table 1). One of the best-studied examples is the mouse Nkx2.5 locus. LacZ reporter assays identified enhancer elements that specifically drove Nkx2.5 expression in different chambers of the hearts, as well as in thyroid, pharynx, and stomach within a 14 kb window around the TSS, revealing previously unappreciated complex enhancer modules underlying the control of cardiac TFs. Similar complexities were seen at genes encoding other cardiac TFs, such as Hand2 (heart and pharyngeal specific enhancers) (McFadden et al., 2000; Charité et al., 2001; Iklé et al., 2012), Mef2c (anterior heart field and somite specific enhancers) (Wang et al., 2001; Dodou et al., 2004), and Gata4 (lateral mesoderm, endocardium, and endoderm specific) (Rojas et al., 2005, 2009; Schachterle et al., 2012). Although limited in number and biased toward proximal gene promoter regions, these studies (and many others) have revealed fundamental principles and mechanisms underlying cardiac gene regulation.

**Establishing Molecular Cascades Regulating Heart Development**

Enhancers represent information hubs that integrate multiple upstream regulatory inputs such as lineage-determining master TFs and signaling effectors. Dissecting the transcription factors that bind to enhancers unveils these direct upstream regulators (Figure 1 and Table 1). By combining motif mutagenesis, gel shift, and transgenic assays, Nkx2.5 enhancer studies revealed that GATA4 and SMAD-mediated BMP signaling directly activated Nkx2.5 expression through multiple enhancer regions (Seacey et al., 1998; Lien et al., 1999, 2002; Liberatore et al., 2002; Brown et al., 2004) (Figure 1). Dissections of Nkx2.5 enhancers in the following years added ISL1, TBX20, MEF2C, and NFAT into direct upstream regulators that collectively drove Nkx2.5 expression in cardiac cells (Takeuchi et al., 2005; Chen and Cao, 2009; Clark et al., 2013). Furthermore, mining known heart enhancers can also lead to discoveries of novel cardiac regulators. For example, MZF1, previously known as a hematopoietic TF, was found to bind to an Nkx2.5 enhancer from *in silico* motif analysis and validated in embryonic stem cell (ESC) differentiation. Overexpression of MZF1 at different stages of cardiac differentiation revealed its novel, stage-dependent roles in cardiogenesis (Doppler et al., 2014).

Through similar enhancer dissection, the upstream signals of many other cardiac TFs have been identified (Table 1 and Figure 1). For example, the lateral mesoderm expression of mouse *Gata4* relies on transcriptional inputs from FOXF1, BMP4, and its autoregulation (Rojas et al., 2005), while its expression in endocardia requires binding of ETS factors such as ETS1 and ERG (Schachterle et al., 2012). The anterior heart field (AHF) expression of Mef2c is positively regulated by GATA4, ISL1, and TBX20 and repressed by TBX1 through an intronic enhancer (Dodou et al., 2004; Takeuchi et al., 2005; Pane et al., 2018). Ventricular expression of Hey2 is dependent on TBX20 and GATA factor binding, but not NK-2 proteins. Summarizing the existing examples, it is clear that GATA factors, which regulate the expression of many other cardiac TFs (NKK2.5, HAND2, HEY2, MEF2C, etc.), sit among the top of the cardiac molecular cascade. Importantly, sustained cardiac expression of GATA itself requires the transcriptional inputs of other cardiac genes such as NKK2.5 and TBX factors, likely establishing a reciprocal feedback loop to maintain the robustness of the cardiac regulatory network.

**Cardiac TF Crosstalk**

Enhancer activation requires the cooperative binding of multiple TFs, therefore studying heart enhancers reveals cooperation and competition between these upstream factors. By co-expressing different combinations of factors together with a specific enhancer, the synergistic effect of factors in activating the enhancer can be revealed by quantitative measures like luciferase assays. Using this type of approach, GATA4 and SMAD1/4 were found to work as mutual co-activators in activating Nkx2.5 expression through a distal enhancer (commonly referred to as the G-S enhancer) (Brown et al., 2004). At another Nkx2.5 enhancer (AR1), GATA binding is indispensable for
TABLE 1 | Functionally characterized enhancer regions near cardiac genes.

| Target genes   | Enhancer length | Genomic position | Expression domain                                                                 | Upstream regulators or function | References                  |
|----------------|-----------------|------------------|------------------------------------------------------------------------------------|---------------------------------|-----------------------------|
| Mouse Nkx2.5   | 14 kb           | 5’ flanking sequence of TSS | cardiac crescent, ventricles, outflow tract, pharynx, thyroid, stomach             | NKX2.5 (negatively regulate this enhancer) | Tanaka et al., 1999         |
| Mouse Nkx2.5   | 4, 3.3 kb       | 5’ flanking sequence of TSS | outflow tract, basal portion of the right ventricle, pharynx, thyroid              |                                 | Tanaka et al., 1999         |
| Mouse Nkx2.5   | 6 kb            | 3’ flanking sequence of TSS | right ventricle                                                                     |                                 | Tanaka et al., 1999         |
| Mouse Nkx2.5   | 8 kb            | [-14, –6 kb] of TSS  | medial wall and inner trabeculae of ventricles                                    |                                 | Tanaka et al., 1999         |
| Mouse Nkx2.5   | 2.1 kb, two     | [-9.4, –7.3 kb] of TSS | endogenous cardiac expression of Nkx2.5                                          | GATA4, MEF2C, NFAT, MZF1         | Searcy et al., 1998; Liberatore et al., 2002; Lien et al., 2002; Takeuchi et al., 2005; Chen and Cao, 2009; Quinodoz et al., 2018 |
| Mouse Nkx2.5   | 505 bp (AR2)    | [–3, –2.5 kb] of TSS  | anterior cardiac crescent, right ventricle, outflow tract, developing spleen, pharyngeal pouches | GATA, SMAD, NFAT, ISL1           |                             |
| Mouse Nkx2.5   | 2, 1.5 kb       | [–10.7, –3.5 kb] of TSS | early heart tube, outflow tract, right ventricle                                   | GATA                            | Reecy et al., 1999          |
| Mouse Nkx2.5   | 237 bp (G-S)    | [–6.2, –5.79 kb] of TSS | cardiac crescent, heart, forebrain                                                | GATA4, SMAD1/4                   | Brown et al., 2004          |
| Mouse Nkx2.5   | 10 kb (FL)      | 5’ flanking sequence of TSS | test in cell lines (10T1/2, P19)                                                  | GATA4, SMAD1/4, TBX20            | Brown et al., 2004; Takeuchi et al., 2005; Chi et al., 2005 |
| Mouse Nkx2.5   | 2.6 kb (UH5)    | [–16, –14 kb] of TSS (estimated) | heart tube, both atria, left ventricle, foreleg                                   |                                 |                             |
| Mouse Nkx2.5   | 7.3 kb (UH6)    | [14, –6 kb] of TSS (estimated) | right ventricle, interventricular septum, atrial ventricular canal               |                                 |                             |
| Chicken Nkx2.5 | 3 kb, 200 bp    | [+976 bp, +3.97 kb, +2.1, +2.3 kb] of TSS | anterior cardiac crescent, outflow tract, right ventricle, pharyngeal arches (test in mouse) | GATA4/5/6, SMAD, YY1            |                             |
| Mouse Gata4    | 4.4 kb (G2)     | [–45.3, –40.9 kb] of TSS | lateral mesoderm                                                                  | FOXF1, GATA4, BMP4              | Rojas et al., 2005          |
| Mouse Gata4    | 1.9 kb (G9)     | 93 kb upstream of TSS | cardiac crescent, linear heart tube, endocardium                                   | EST factors (ETS1, ERG)         | Schachterle et al., 2012   |
| Zebrafish gata4| 14.8, 12 kb     | 5’ flanking sequence of TSS | lateral plate mesoderm, both atrium and ventricle                               |                                 | Heicklen-Klein and Evans, 2004 |
| Zebrafish gata4| 7.8, 5.5 kb     | 5’ flanking sequence of TSS | ventricle and the bulboventricular valve                                         |                                 | Heicklen-Klein and Evans, 2004 |
| Zebrafish gata4| 3 kb (DR1), 1.3 kb (DR1A) | [–11, –8 kb] of TSS | lateral plate mesoderm, both atrium and ventricle                               | TBX                             | Heicklen-Klein and Evans, 2004 |
| Chicken GATA5  | 500 bp          | [–5, –4.5 kb] of TSS | cardiac crescent, septum trans-versum and epicardium, ventricle, AV canal (test in mice) |                                 | MacNeill et al., 2000       |
| Mouse Gata6    | 6.8, 1.8 kb     | [–4.3, +2.5 kb, [–4.3, –2.5 kb] of TSS | cardiac crescent, high expression in outflow tract                               | NKX2.5                          | Molkanatin et al., 2000    |
| Mouse Gata6    | 1.4 kb          | 6.2 kb upstream of TSS | cardiac crescent, high expression in the outflow tract (test in mouse)           | NKX2.5                          | Davis et al., 2000         |
| Mouse Gata6    | 10 kb           | [–9.2, +0.8 kb] of TSS | cardiac specific (test in mice)                                                   | Retinoic acid                   | He and Burch, 1997; Davis et al., 2001 |
| Mouse Gata6    | 2.3, 1.5 kb     | [–1.5, +0.8 kb, [–1.5 kb, 0] of TSS | posterior region of the heart field, atrioventricular conduction system (test in mice) |                                 |                             |
| Mouse Hand2    | 1.5 kb          | [–4.2, –2.7 kb] of TSS | atrioventricular conduction system (test in mice)                                 | GATA                            | Adamo et al., 2004         |

(Continued)
the transcriptional activation mediated by NFAT, likely through cooperative binding (Chen and Cao, 2009). Besides cooperativity, competitive binding between different TFs at heart enhancers can also play an important role in cardiac lineage specification. For example, the two homeodomain TFs, NKX2.5 and ISL1 compete also play an important role in cardiac lineage specification. For cooperative binding between different TFs at heart enhancers can (Chen and Cao, 2009). Besides cooperativity, the transcriptional activation mediated by NFAT, likely through homozygous enhancer knockout, contain SNPs associated with heart rate.

### Putting Enhancers to Work

Besides providing direct evidence for building cardiac transcriptional networks, validated cardiac enhancers also frequently serve as genetic tools to label a specific cardiac population of interest for developmental studies. Transgenic mice in which Cre recombinase expression is driven by the 

| Target genes | Enhancer length | Genomic position | Expression domain | Upstream regulators or function | References |
|--------------|-----------------|------------------|-------------------|-------------------------------|------------|
| Mouse Mef2c  | 6.39 kb, 449 bp  | [+16.3, +22.5 kb] of TSS | anterior (second) heart field | GATA4, ISL1, NKX2.5, TBX20, TBX1 (negative regulator) | Dodou et al., 2004; Takeuchi et al., 2005; Caputo et al., 2015; Pane et al., 2018 |
| Mouse Hey2  | 2.5, 1.6 kb, 649 bp | 211 kb upstream of TSS | cardiac crescent, ventricle and outflow tract | TBX20, GATA4 | Ihara et al., 2020 |
| Zebrafish hey2 | 626 bp | 24 kb upstream of TSS | distal linear heart tube, ventricle, outflow tract | FOX (likely FOXC1 or FOXC2) | Gibb et al., 2018; Yuan et al., 2018 |
| Mouse Tbx1  | 200 bp (require another non-cardiac element) | [−12.8, −12.6 kb] of TSS | second heart field, right ventricle, outflow tract, pulmonary trunk, and pulmonary valves | | Maeda et al., 2006 |
| Human TBX5  | 368 bp (enhancer 2) | 380 kb downstream of TSS | both ventricles and atria | Harbor a CHD-associated variant | Smemo et al., 2012 |
| Human TBX5  | 3.5 kb (enhancer 9) | 140 kb downstream of TSS | ventricles, interventricular septum, atrioventricular canal | | Smemo et al., 2012 |
| Human TBX5  | 5 kb (enhancer 16) | 9 kb upstream | ventricles, interventricular septum, atrioventricular canal, and weakly in atria | | Smemo et al., 2012 |
| Mouse Isl1  | 2.9 kb | 120 kb downstream | embryonic and adult sinoatrial node (SAN) | SAN hypoplasia and sinus arrhythmia in enhancer knockout, contain SNPs associated with heart rate | Galang et al., 2020 |
| Mouse Fgf8  | 900 bp | [−5.4, −4.5 kb] of TSS | outflow tract, pharyngeal arches | TBX1 | Hu et al., 2004 |
| Mouse Fgf10  | 1.7 kb | [+44, +64 kb] of TSS | anterior second heart field, pharyngeal mesoderm | TBX1, NKX2.5 (negative), ISL1 | Watanabe et al., 2012 |
| Mouse Sry  | 1 kb, 541 bp | 3′ UTR sequence | cardiac crescent, heart tube, tail | TBX2, TBX5, TIP60 | Barron et al., 2005 |

| References |
|------------|
| Source: Smemo et al., 2012 |

In sum, deeply dissecting cardiac enhancers reveals both molecular tools for visualizing, isolating, and manipulating cardiac populations as well as cis- and trans-regulatory mechanisms that control cardiac gene expression.
UNMASKING HEART ENHANCERS WITH COMPARATIVE AND FUNCTIONAL GENOMICS

Enhancer Hunting: Tools of the Trade

Comparative genomics has long been used to identify putative enhancer regions (Table 1; Aparicio et al., 1995). Such comparative approaches are based on the assumption that functionally relevant enhancer sequences will be under negative selection and will thus show higher sequence constraints than non-functional regions. This assumption is supported by the genome-wide identification of conserved non-coding elements (CNEs) and the following discoveries that many CNEs work as developmental enhancers (Nobrega et al., 2003; Bejerano et al., 2004; Johnson et al., 2004; de la Calle-Mustienes et al., 2005; Shin et al., 2005; Woolfe et al., 2005; Pennacchio et al., 2006). Substantial work using a variety of approaches including transitive alignment (Hiller et al., 2013; Braasch et al., 2016), ancestral reconstruction (Hiller et al., 2013), and conserved microsynteny (Irimia et al., 2012; Clément et al., 2020; Wong et al., 2020) have further enhanced our ability to detect more distantly related conserved non-coding elements.

Although CNEs are enriched for developmental enhancers, the vast majority of enhancers appear to evolve more rapidly, with many being lineage- or species-specific. This feature has been demonstrated in many different tissues and cell types and in both vertebrates and invertebrates (Odom et al., 2007; Kunarso et al., 2010; Schmidt et al., 2010b; Mikkelsen et al., 2010; Cotney et al., 2013; Paris et al., 2013; Arnold et al., 2014; Villar et al., 2015). Although enhancers in different tissues or at different developmental stages may be under varied selection pressures (Blow et al., 2010; Nord et al., 2013; Visel et al., 2013), rapid evolution is an overall feature of enhancer sequences, which suggests that many enhancers would be missed in detection approaches based on sequence conservation alone.

Over the past 15 years, large scale genomic assays have enabled enhancer discoveries at an unprecedented scale (Table 2). In particular, chromatin immunoprecipitation with high-throughput sequencing (ChIP-seq) can locate enhancers by profiling the co-occupancy of lineage-specific TFs, binding of co-factors, or post-transcriptional modifications that marks active enhancers (reviewed in Buecker and Wysocka, 2012). As ChIP-seq requires large numbers of input cells, which is often difficult to obtain from early embryonic tissues, many low input ChIP methods (O’Neill et al., 2006; Brind’Amour et al., 2015) and alternative strategies, such as enzyme-tethering based approaches have been established (e.g., CUT&RUN, CUT&Tag, and CUTAC) (Skene and Henikoff, 2017; Kaya-Okur et al., 2019; Meers et al., 2019; Henikoff et al., 2020).

Chromatin accessibility profiling provides a comprehensive view of the candidate regions most likely to harbor CREs, making them arguably the most widely used assay to identify putative enhancers (Thurman et al., 2012; Buenrostro et al., 2013; Vierstra et al., 2014, 2020; Corces et al., 2017). Since active enhancers are transcribed bidirectionally to produce eRNA, nascent RNA sequencing technologies, specifically the run-on assays (GRO-seq, PRO-seq, ChRO-seq, etc.), can be used as a direct readout of enhancer activity. Furthermore, when coupled with chromatin accessibility assays (i.e., ATAC-seq), run-on assays can distinguish active enhancers (producing bi-directional RNAs) from other CREs such as CTCF bound insulators (reviewed in Wissink et al., 2019).

After discovering a distal putative enhancer, one of the most pressing questions is to discover what gene or genes it associates within a cell type and condition of interest. To address this, chromosome conformation capture (3C) based assays (including 4C, 5C, HiChIP, promoter capture Hi-C, and Hi-C) are commonly used to characterize enhancer-promoter interactions (Denker and De Laat, 2016; Fang et al., 2016; Mumbach et al., 2016). Capture Hi-C approaches, such as promoter-capture Hi-C and HiCap (Mifsud et al., 2015; Sahlén et al., 2015; Schoenfelder et al., 2015), are increasingly being used to reveal promoter-centric chromatin interactions at high resolution. Capture-based methods that target putative enhancer regions, such as those discovered by DNase-seq (Sönmez et al., 2020), could also be used for ‘enhancer-capture’ Hi-C. Naturally, the choice of 3C-based methods depends on the research question and practical considerations such as the quantity of sample material, genome size, capture probe availability, and sequencing costs.

These widely used genome-scale assays, each with their own strengths (Table 2), continue to reveal new insights into enhancer location, activity and function. The increasing number of high-quality datasets are also creating new opportunities and challenges for integrative data analysis that will further expand our understanding of metazoan heart development and human disease.

Heart Enhancers: From Genome-Wide Mapping to Metazoan Regulatory Logic

The development of ChIP-chip, ChIP-seq, and other genomic techniques has enabled genome-wide enhancer discoveries and analysis of distinct cardiac samples obtained from diverse model systems (Table 3). Pioneering studies in Drosophila using ChIP-chip against master regulators (Twi, Tin, Mef2, Bag, Bin, Doc, and Pnr) and signaling effectors (dTCF and pMad) required for the specification of cardiac mesoderm revealed fundamental principles of combinatorial TF binding dynamics and TF-signaling interactions at cardiac enhancers (Zinzen et al., 2009; Junion et al., 2012). These Drosophila cardiac TF mapping studies, together with a comparative analysis of Twi, Tin, Mef2, Bin, and Bap in two distant Drosophila species, underscore the conserved presence of combinatorial TF binding, even when the underlying DNA sequence has changed (Khoueiry et al., 2017). The Junion et al. (2012) study led to a "transcription factor collective" model of TF binding where TFs use both protein-DNA and protein–protein interactions to regulate gene expression (reviewed by Spitz and Furlong, 2012), which was later supported by the comparative Khoueiry et al. (2017) study.

To demarcate the location of putative enhancers active in embryonic and adult hearts, pioneering mammalian studies performed ChIP-seq for the histone acetyltransferase EP300 and...
However, it is still unclear whether the TFs defines mammalian heart enhancers (He et al., 2011; Zhou et al., 2017). These studies fit the “TF collective model” proposed for Drosophila, the “billboard model (Arnosti and Kulkarni, 2005), “ in which specific sets of TFs are recruited to enhancers with flexible motif grammar; or a mixture of models (Long et al., 2016). For example, the importance of heterotypic interactions between mouse TBX5 and NKK2-5 was demonstrated using co-crystal structure together with DNA, as well as ChIP-exo experiments (Luna-Zurita et al., 2016). Intriguingly, the genetic loss of either Tbx5 or Nkx2-5 led to ectopic interactions of the other remaining TF. Unlike the more flexible “TF collective” or “billboard” models, TBX5 and NKK2-5 co-occupancy highlighted in this study featured preferred motif arrangements. Most recently, a novel single molecule footprinting (SMF) method was used to detect factor-centric chromatin interactions similar to CHIA-PET but require 10-fold to 100-fold fewer cells, also more robust and less time-consuming (Fang et al., 2016; Mumbach et al., 2016).

| Method category | Method strategy | Description | References |
|-----------------|-----------------|-------------|------------|
| Enzyme tethering ChIP alternative | CUT&RUN (pA-MNase fusion protein) | Detects factor-centric chromatin interactions similar to CHIA-PET but require 10-fold to 100-fold fewer cells, also more robust and less time-consuming | Kaya-Okur et al., 2019; Henikoff et al., 2020 |
| Accessible chromatin profiling | HiC | Maps genome-wide chromatin contacts (‘all-to-all’); requires substantial sequencing to reveal local enhancer-promoter interactions | Lieberman-Aiden et al., 2009 |
| Chromosome conformation capture | Hi-C | Maps genome-wide chromatin contacts (‘all-to-all’); requires substantial sequencing to reveal local enhancer-promoter interactions | Lieberman-Aiden et al., 2009 |
| Nascent RNA sequencing run-on assays | PRO-seq | Refined version of GRO-seq that uses biotinylated nucleotide to reach nucleotide-resolution, low background, and large dynamic ranges | Kwa et al., 2013; Core et al., 2014 |
| | GRO-seq | Detect actively transcribed eRNAs which is a hallmark of active enhancers | Core et al., 2008 |
| | ATAC-seq | Simple and robust method that requires low cell numbers, widely applied; can be used on frozen sections; produces a comprehensive list of where CREs may be located. | Buenrostro et al., 2013; Corces et al., 2017 |
| | DNase-seq | High quality TF footprint scan can be generated. | Thurman et al., 2012; Vierstra et al., 2014, 2020 |
| | CUT&Tag (pA-TnS) | Similar to CUT&RUN with a simpler barcoding step; streamlined workflow in a single tube; works on low cell numbers or even single cells | Kaya-Okur et al., 2019; Henikoff et al., 2020 |
| | CUTAC (pA-TnS, low salt) | Similar to CUT&Tag with a small modification that detects accessible chromatin in parallel with adjacent histone modifications | Henikoff et al., 2020 |
| | CUT&RUN (pA-MNase fusion protein) | Unified in-situ procedure, requires lower cell numbers (~100 for histone modification) and less sequencing reads | Skene and Henikoff, 2017; Meers et al., 2019 |
| | CUT&Tag (pA-TnS) | Similar to CUT&Tag with a small modification that detects accessible chromatin in parallel with adjacent histone modifications | Henikoff et al., 2020 |
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**TABLE 3** | Genome-wide metazoan heart enhancer profiling datasets generated using chromatin immunoprecipitation of post translational histone modifications, transcription factors, and cofactors.

| Method            | Species          | Factor                        | Sample                             | Condition                                                                 | Stage                                      | References                          |
|-------------------|------------------|-------------------------------|------------------------------------|--------------------------------------------------------------------------|--------------------------------------------|-------------------------------------|
| BiTS-ChIP-Seq     | Drosophila       | H3, H3K4me3, H3K4me1, H3K27ac, H3K27me3, H3K36me3, H3K79me3 | Mesoderm                          | WT                                                                        | stages 10–11 (6–8 h AEL, cardiac mesoderm specified) | Bonn et al., 2012                   |
| ChIP-seq          | Zebrafish        | H3.3                          | myl7::GFP+cardiomyocytes           | Uninjured, 14 days post ablation, 7 days post Nrg1 treatment             | Adult                                      | Goldman et al., 2017                |
| ChIP-seq          | Zebrafish        | H3K27ac                       | myl7::GFP+cardiomyocytes           | Uninjured, 14 days post ablation                                         | Adult                                      | Wamstad et al., 2012                |
| ChIP-seq          | Mouse            | H3K27ac, H3K4me1, H3K4me3, H3K27me3 | ESCs, ESC-differentiated cells     | WT                                                                        | ESCs, mesoderm, cardiac precursors, cardiomyocytes | Nord et al., 2013                   |
| ChIP-seq          | Mouse            | H3K27ac                       | Hearts                            | WT                                                                        | E11.5, E14.5, E17.5, P0, P7, P21, P56 | He et al., 2014                     |
| ChIP-seq          | Mouse            | H3K4me1, H3K27me3, H3K4me3    | Ventricle                         | WT                                                                        | E12.5 and adult                            | Zhou et al., 2017                   |
| ChIP-seq          | Mouse            | H3K27ac                       | Ventricle                         | WT, GATA4 KO                                                             | E12.5 (WT, GATA4 KO), adult (normal)       | Hashimoto et al., 2019              |
| ChIP-seq          | Mouse            | H3K27ac                       | Heart                             | WT                                                                        | E12.5                                      |                                     |
| ChIP-seq          | Mouse            | H3K27ac                       | iCLM (induced cardiac-like myocytes) reprogrammed from MEF | Transfected with GMT, GHMT, AGHMT or mock control                         | Day 2 and 7 in reprogramming               |                                     |
| ChIP-seq          | Mouse            | H3K27ac                       | iCLM (induced cardiac-like myocytes) reprogrammed from MEF | Transfected with single factors                                          | Day 2 in reprogramming                     |                                     |
| ChIP-seq          | Human            | H3K27ac                       | Ventricles, atrium ESCs, ESC-differentiated cells | WT                                                                        | P4                                         | Paige et al., 2012                  |
| ChIP-seq          | Human            | H3K4me3, H3K27me3, H3K36me3   | WT                                | WT                                                                        | pluripotent cells, mesodermal progenitors, specified tripotential cardiovascular progenitors, committed cardiovascular cells, definitive cardiovascular cells | Ang et al., 2016                   |
| ChIP-seq          | Human            | H3K4me3, H3K27ac, H3K27me3    | PSC-differentiated cells           | WT, GATA4_G206S                                                           | IPSC-derived cardiomyocytes                | Gilsbach et al., 2018               |
| ChIP-seq          | Human            | H3K27ac, H3K9ac, H3K4me1, H3K4me3, H3K27me3, H3K36me3 | Left ventricle                    | Healthy donor and patients with heart failure                             | fetal, infant, adult (non-failing and failing heart) | Spurrell et al., 2019               |
| ChIP-seq          | Human            | H3K27ac                       | Left ventricle                    | healthy donors and patients with dilated cardiomyopathy                  | Adult                                      |                                     |
| ChIP-seq          | Human            | H3K27ac                       | ESCs, ESC-differentiated cells     | WT                                                                        | ESCs, mesodermal cells, cardiac mesodermal cells, cardiac progenitors, primitive cardiomyocytes, and ventricular cardiomyocytes | Zhang et al., 2019                  |
| ChIP-seq          | Human            | H3K4me1, H3K4me2, H3K4me3, H3K27ac, H3K27me3, H3K36me3 | Heart                             | Healthy donor                                                             | CS13, CS14, CS16, CS17, CS18, CS19, CS20, CS21, CS23 (Carnegie stage, corresponding to PCW 4–8) | Vanourenhove et al., 2020            |

(Continued)
### TABLE 3 | Continued

| Method       | Species            | Factor                           | Sample               | Condition                        | Stage                                                                 | References          |
|--------------|--------------------|----------------------------------|----------------------|----------------------------------|------------------------------------------------------------------------|---------------------|
| ChIP-chip    | Drosophila         | Twist, Tinman (Nkx2.5)           | Whole embryo         | WT                               | Stage 5–7, stage 8–9 (dorsal mesoderm specified), stage 10–11 (cardiac mesoderm specified) | Zinzen et al., 2009 |
| ChIP-chip    | Drosophila         | MeF2                             | Whole embryo         | WT                               | Stage 5–7, stage 8–9, stage 10–11                                      |                    |
| ChIP-chip    | Drosophila         | Bagpipe                          | Whole embryo         | WT                               | Stage 10–11                                                             |                    |
| ChIP-chip    | Drosophila         | Biniou                           | Whole embryo         | WT                               | Stage 10–11, stage 12–13, stage 13–15                                  |                    |
| ChIP-chip    | Drosophila         | Dorsocross, Pannier, dTCF, and pMad | Whole embryo         | WT                               | Stage 8–9, stage 10–11                                                  | Junion et al., 2012 |
| BITS-ChIP-seq| Drosophila         | MeF2, Rpb3-Pol II                 | Mesoderm             | WT                               | stages 10–11                                                            | Bonn et al., 2012  |
| ChIP-seq     | Drosophila         | MeF2                             | Whole embryo         | WT                               | Stage 5–7, stage 8–9, stage 10–11                                      | Khoueiry et al., 2017 |
| ChIP-seq     | Drosophila         | Twist and Drosophila melanogaster and Drosophila virilis | Whole embryo         | WT                               | Stage 8–9, stage 10–11                                                  |                    |
| ChIP-seq     | Drosophila         | Twist                            | Whole embryo         | WT                               | Stage 5–7, stage 8–9, stage 10–11, stage 12–13, stage 13–15            |                    |
| ChIP-seq     | Drosophila         | Tinman                           | Whole embryo         | WT                               | Stage 8–9, stage 10–11                                                  |                    |
| ChIP-seq     | Drosophila         | Bagpipe                          | Whole embryo         | WT                               | Stage 10–11, stage 12–13, stage 13–15                                  |                    |
| ChIP-seq     | Mouse              | P300                             | Heart                | WT                               | E11.5                                                                   | Blow et al., 2010   |
| ChIP-seq     | Mouse              | P300                             | Heart                | WT                               | P2                                                                      | May et al., 2012    |
| ChIP-seq     | Mouse              | GATA4 (flag or biotin-tagged)    | Ventricle            | WT                               | E12.5                                                                   | He et al., 2014     |
| ChIP-seq     | Mouse              | GATA4 (flag or biotin epitope-tagged) | Ventricle           | Normal, banding (surgically placed ligature around the aorta), sham | Adult                      |                    |
| ChIP-seq     | Mouse              | GATA4, TBX3, Nkx2.5, P300         | Heart                | WT                               | Adult                                                                   | van den Boogaard et al., 2012 |
| ChIP-seq     | Mouse              | HAND2 (flag-tagged)              | Limb bud, hearts, branchial arches | WT                               | E10.5                                                                   | Osterwalder et al., 2014 |
| ChIP-seq     | Mouse              | Nkx2.5                           | Heart                | WT                               | E11.5                                                                   | Dupays et al., 2015 |
| ChIP-exo     | Mouse              | GATA4, Nkx2.5, and TBX5           | ESCs, ESC-differentiated cells | WT, Nkx2.5 KO, TBX5 KO, double KO | cardiac precursors and cardiomyocytes                                  | Luna-Zurita et al., 2016 |
| ChIP-seq     | Mouse              | P300 (biotin-tagged)             | Heart                | WT                               | E12.5, Adult                                                            | Zhou et al., 2017   |
| ChIP-seq     | Mouse              | P300 (biotin-tagged)             | Endocardial and endothelial cells in the heart | WT                               | Adult                                                                   |                    |
| ChIP-seq     | Mouse              | CTCF                             | Left ventricle (isolated cardiomyocytes) | WT, CTCF KO | Adult                                                                   | Rosa-Garrido et al., 2017 |
| ChIP-seq     | Mouse              | HAND2 (flag-tagged)              | Heart                | WT                               | E10.5                                                                   | Laurent et al., 2017 |
| ChIP-seq     | Mouse              | TBX20 (GFP-tagged)               | Heart                | WT                               | E11.5                                                                   | Boogerd et al., 2017 |

(Continued)
to ascertain TF co-occupancy in mouse embryonic stem cells (Sönmezer et al., 2020). In this study, simultaneous TF binding did not depend on the identity of the TFs involved, and the co-occupancy of TFs on chromatin lacked of strict motif organization, which the authors proposed agreed with the “billboard model” (Sönmezer et al., 2020). Indeed, comparative approaches using this SMF method to study enhancer logic during metazoan cardiac development will be insightful for both learning general principles governing enhancer regulation as well as the biologically important exceptions that define key physiological processes.

To study cardiac enhancer dynamics across multiple stages of in vitro cardiac differentiation or in vivo development, several studies from individual labs as well as consortia, have utilized robust genome-wide assays that do not rely on mapping specific transcription factors, namely ChIP-seq for histone modifications (Paige et al., 2012; Wamstad et al., 2012; Nord et al., 2013; Vanoudenhove et al., 2020), and DNase-seq and ATAC-seq for chromatin accessibility (Bertero et al., 2019; Gorkin et al., 2020; Meuleman et al., 2020). These studies revealed highly dynamic chromatin states accompanying cardiac differentiation and development. Specifically, ATAC-seq is widely used on precious in vivo cardiac samples to identify genomic regions that are enriched for TF binding and functional enhancer elements (jia et al., 2018; Yuan et al., 2018; Pawlak et al., 2019; Racioppi et al., 2019). Recently, accessible chromatin profiling has also enabled the discovery of enhancers specific to cardiac subpopulations, such as pacemaker cells (Galang et al., 2020; van Eif et al., 2020) and endocardial populations (Boogerd et al., 2017).

Functional insights into cardiac enhancer regions continue to be made by studying TF occupancy and chromatin states upon the perturbation of cardiac TFs or signaling pathways in multiple organisms (e.g., Gata4, gata5, Nkx2.5, Tbx5/tbx5, Tbx20, Hand2/hand2, Isl1, Foxf, Fgfr, Mek, and Ras) (He et al., 2014; Luna-Zurita et al., 2016; Boogerd et al., 2017; Jia et al., 2018; Pawlak et al., 2019; Racioppi et al., 2019), as well as in a human congenital heart disease (CHD) model (cardiomyocytes with a disease-associated missense mutation of GATA4) (Ang et al., 2016). These studies reveal the master regulatory roles of cardiac TFs at the chromatin level. For example, GATA4 is essential for establishing open chromatin, promoting active epigenetic modification (H3K27ac) and recruiting TBX5 to the proper cardiac enhancers (He et al., 2014; Ang et al., 2016). On the other hand, TBX5 and NKK2.5 are important for preventing ectopic binding of GATA4 during cardiac differentiation, highlighting the importance of interdependent
co-occupancy of these cardiac TFs in precisely controlling cardiac gene expression (Luna-Zurita et al., 2016). This interdependent co-occupancy is also essential in cardiac reprogramming, as only co-expression of cardiac factor cocktails (GATA4, HAND2, TBX5, MEF2C, etc.), but not single-TF overexpression, can lead to robust cardiac TF occupancy to reprogramming enhancers (Hashimoto et al., 2019).

Heart Enhancers in Space: Chromatin Interactions and Architectures

Heart enhancer activity not only requires proper TF binding, but is under the control of local chromatin interactions and higher-order chromatin architectures. Several groups have conducted promoter capture Hi-C in ESC/iPSC-derived cardiomyocytes or adult hearts to map enhancer-promoter interactions (Choy et al., 2018; Montefiori et al., 2018; Jung et al., 2019). These promoter capture Hi-C studies identified potential target genes for a substantial fraction of candidate heart enhancers. Interestingly, on average 25–35% of cell chromatin regions per gene and 40–60% of distal regions interacting with more than one gene. Hi-C has also been recently used to profile high-order chromatin architectures such as TADs and compartments across closely sampled time points during the differentiation from stem cells to cardiomyocytes (Bertero et al., 2019; Zhang et al., 2019). These studies showed extensive rearrangement of chromatin architectures during cardiac cell differentiation, with 19% genome switching compartments and 20–40% of TADs being stage-specific. Integrated analyses based on these datasets also revealed important regulatory mechanisms and unknown regulators in heart development. For example, Bertero et al. (2019) detected spatial coalescence of multiple cardiac genes from different chromosomes. This coalescence formed a trans-interacting chromatin domain that recruited the muscle-specific splicing factor RBM20 for efficient pre-mRNA splicing (Bertero et al., 2019).

The importance of chromatin interactions and architecture in heart development and function is also revealed by the essentiality of genome organizing factors such as CTCF and the cohesin complex. CTCF knock-out in cardiac progenitor cells leads to severe defects in cardiac cell maturation due to the disruption of enhancer-promoter interaction and subsequent misregulation of cardiac genes (Gomez-Velazquez et al., 2017). In the adult heart, CTCF depletion is sufficient to induce pathological consequences that are very similar to heart failure (Rosa-Garrido et al., 2017). Knock-out of Stag2 (which encodes a cohesin subunit) in embryonic mice leads to lethality by E10.5 due to severe morphogenesis defects in SHF-derived structures (right ventricle, outflow tract and septation), however, due to severe morphogenesis defects in SHF-derived structures (right ventricle, outflow tract and septation), but not single-TF overexpression, can lead to robust cardiac TF occupancy to reprogramming enhancers (Hashimoto et al., 2019).

Enhancing Enhancers With Enhancer-Associated RNAs

Upon activation, many enhancers are transcribed into non-coding RNAs, which are broadly referred to as enhancer RNAs (eRNAs). The expression of eRNAs is well correlated with their putative target gene expression (Kim et al., 2010; Kaikkonen et al., 2013; Li et al., 2013; Andersson et al., 2014; Arner et al., 2015). eRNAs may not only serve as hallmarks of enhancer activation, but also exert important functions in driving target gene expression by promoting chromatin accessibility (Mousavi et al., 2013), mediating enhancer-promoter interaction (Lai et al., 2013; Li et al., 2013; Hsieh et al., 2014), regulating chromatin remodeling (Kaikkonen et al., 2013), and facilitating PolII pause-release at promoters (Schaukowitch et al., 2014; Shli et al., 2017). However, for the vast majority of eRNAs, it remains unclear whether they are simply by-products of enhancer transcription or whether they possess functional roles based on the transcriptional process itself, or through additional molecular interactions in cis or in trans (reviewed in Li et al., 2016; Arnold et al., 2020).

Though early discoveries described eRNAs as short, non-polyadenylated, bidirectionally transcribed RNAs (Kim et al., 2010; Andersson et al., 2014), a diverse group of molecules with other structures (long, polyadenylated, or unidirectionally transcribed) have been attributed to eRNAs (Koch et al., 2011; Kaikkonen et al., 2013; Li et al., 2013; Alvarez-Dominguez et al., 2017). The structure and functional similarities between some eRNAs and cis-acting long non-coding RNAs (lncRNAs) have raised an emerging concept that they represent overlapping categories of regulatory non-coding RNAs (lncRNAs) that have raised an emerging concept that they represent overlapping categories of regulatory non-coding RNAs (lncRNAs) (Espinosa, 2016; Paralkar et al., 2016; Arnold et al., 2020; Gil and Ulitsky, 2020).

The roles of eRNAs and lncRNAs in the contexts of heart development have been explored by many studies (Grote et al., 2013; Klattenhoff et al., 2013; Ounzain et al., 2014, 2015; Anderson et al., 2016; Alexanian et al., 2017; Yang et al., 2017; Turton et al., 2019; Nicole Ritter et al., 2019). Given the challenge in categorizing these ncRNAs, we consider all ncRNAs that are associated with heart enhancers and discuss the different ways through which they may regulate heart development using two examples: (1) The ncRNA transcript itself is involved in target gene regulation. For example, using anti-sense mediated RNA knockdown, Yang et al. (2017) showed that the expression of Ryr2, a TBX5 target that is critical for maintaining cardiac rhythm, depends on a novel TBX5-dependent eRNA, RACER; and (2) Instead of the ncRNA molecule, it is the transcriptional activity of the ncRNA locus that appears to be important for controlling the target genes. Two such ncRNAs come from the Hand2 locus, upperhand (Uph) (Anderson et al., 2016) and handsdown (Hdnn) (Nicole Ritter et al., 2019). Particularly, the


Hand2 locus interacts with the Hand2 promoter and putative cardiac enhancers, suggesting it may regulate Hand2 expression via direct chromatin interaction, reminiscent of CREs (Nicole Ritter et al., 2019). The transcription of Uph over a cardiac enhancer upstream of Hand2 allows the binding of GATA4 and deposition of H3K27ac to this enhancer (Anderson et al., 2016). Together, these examples showcase a few models of the complex interactions between enhancers and the ncRNAs associated with them.

The field of enhancer-associated ncRNAs in heart development has many unanswered questions. Future studies that use chromatin run-on assays (GRO-seq, PRO-seq) or generate deeply sequenced RNA-seq datasets coupled with enhancer annotations should help to understand the dynamic changes of eRNAs in development. Functional experiments such as those use RNA targeting Cas protein (Cas13) (Abudayyeh et al., 2017), shRNA (Lambeth and Smith, 2013), or antisense oligonucleotide-mediated knockdown (Dias and Stein, 2002) will also be essential for teasing out the roles of enhancer-associated ncRNAs in gene-regulation independent of the enhancer elements themselves.

Heart Enhancers: Keeping Track of Time

As the activity of enhancers are not only tissue-specific but also stage-specific, it is important to obtain high-resolution temporal profiles of heart enhancers to truly understand their function. This is specifically highlighted by the in vitro cardiac differentiation study from Wamstad et al. (2012), which showed that enhancers active in ESC, mesoderm progenitors, cardiac progenitors, and cardiomyocytes were largely non-overlapping (Wamstad et al., 2012). Consistently, Luna-Zurita et al. (2016) discovered thousands of GATA4, NKX2.5, and TBX5 binding sites were specific to either cardiac progenitor cells or cardiomyocytes. Similar results have also been reported for in vivo development, for example, 80% of the GATA4 binding sites in fetal heart are not occupied by GATA4 in adult heart (He et al., 2014).

Since the heart is the first organ formed in embryogenesis, the embryonic stage that is required to capture the initial phase of cardiogenesis is especially early in development, and is likely during early gastrulation (Scott, 2012; Devine et al., 2014; Lescroart et al., 2014). Though heart enhancers have been extensively characterized across many developmental stages in various species [such as Nord et al. (2013) and Vanoudenhove et al. (2020) and many others in Tables 3–5], there is a paucity of datasets that characterize enhancers active at the initial stage of vertebrate heart development, such as the transition from mesoderm progenitors to cardiac lineages. The majority of in vivo studies in vertebrates used relatively mature cardiac samples, including embryonic hearts with defined chamber structures (e.g., E10.5 and onward in mice) or postnatal heart tissues (Tables 3–5). As these stages are later than when cardiac lineage commitment occurs, these studies may not capture the enhancers that specifically drive early cardiogenesis.

A few recent in vivo studies confirm the observations made from in vitro differentiation that enhancer-associated chromatin states are highly dynamic, especially during early cardiac lineage specification. A recent study that profiled mouse Nkx2.5+ cardiac progenitor cells revealed major changes in chromatin accessibility between E7.5 and E8.5 but only minor differences between E8.5 and E9.5 (Jia et al., 2018). This suggests that early lineage fate transitions may be accompanied by major changes of chromatin states, which become more stabilized in committed cell types. Similar trends are observed in cardiopharyngeal lineage specification in the tunicate Ciona, in which most significant chromatin changes occur between the transition from mesoderm progenitors to cardiopharyngeal progenitors compared to later stages (Racioppi et al., 2019). These examples reveal intriguing dynamics of the enhancers involved in early cardiac lineage decisions, however, much remains to be explored. Filling this knowledge gap, especially in the context of developing embryos, can bring valuable insights into key cellular events in early cardiogenesis.

Evolutionary Mysteries of Heart Enhancers

Intriguing results have emerged from evolutionary studies of heart enhancers. Although the TFs controlling heart enhancers are highly conserved, validated heart enhancers show weak DNA constraint compared to brain enhancers identified at the same developmental stage (E11.5) (Blow et al., 2010). For instance, only 6% of the candidate heart enhancers were deemed to possess high DNA constraint (phastCon score > 600) compared to 44% of forebrain, 39% of midbrain, and 30% of limb enhancers. This could be in part due to the fact that molecularly, the brain seems to be a more conserved organ in terms of the low proportion of positively select genes, old phylogenetic ages of the transcriptomes, and the low percentage of genes showing trajectory changes between different species (Cardoso-Moreira et al., 2019).

It remains an open and intriguing question how heart enhancers that lack evolutionary conservation work together with many conserved cardiac TFs to orchestrate the development of the heart. Several reasons may contribute to this phenomenon. First, it has been demonstrated by many studies that enhancers are rapidly evolving with pervasive turnovers of TF binding sites (TFBSs) (Kunarso et al., 2010; Mikkelsen et al., 2010; Schmidt et al., 2010b; Cotney et al., 2013; Paris et al., 2013; Arnold et al., 2014; Ballester et al., 2014; Villar et al., 2015; Khoueiry et al., 2017). The rapid changes in the sequence, orientation, spacing and numbers of TFBSs within enhancers may not necessarily alter the functional roles of enhancers but do make it hard to detect enhancer sequence homology via genomic sequence alignment. As a consequence, some functionally conserved enhancers will not share detectable sequence homology. A recent and striking example is a sponge Islet enhancer, which drives expression that overlaps endogenous islet gene (isl2a) expression in zebrafish, despite the absence of homologous sequence in the vertebrate genomes. Nevertheless, enhancers with similar TFBS compositions can be found in human and mouse ISLET/Islet regions and their activities resemble that of the sponge enhancer in zebrafish (Wong et al., 2020). A similar strategy based on motif composition also
identified conserved brain enhancers between chordates and hemichordates, which would not have been detected by sequence alignment alone (Yao et al., 2016). These two examples and many others (Fisher et al., 2006; Hare et al., 2008; Friedli et al., 2010; Chatterjee et al., 2011) indicate that a grammar more flexible than strict sequence conservation is used in some enhancers to produce conserved transcriptional “output.” Overall, the discordance between sequence and functional conservation may account for a significant portion of the weakly conserved heart enhancers.

Second, an increasing number of studies indicate that the conservation of enhancers active in early embryonic development follows an hour-glass like pattern (Bogdanovic et al., 2012; Bogdanović et al., 2016; Liu et al., 2020) similar to that of transcriptomes (Irie and Sehara-Fujisawa, 2007; Domazet-Lošo and Tautz, 2010; Kalinka et al., 2010; Irie and Kuratani, 2011; Yanai et al., 2011). However, much less is known about “phylotypic enhancers” that presumably are established prior to organogenesis to set up conserved vertebrate gene expression patterns. A temporal study of developmental enhancers compared the H3K27ac (a mark of active enhancers) profiles across the development of three mouse tissues (heart, brain, and liver) from ESC to adults (Nord et al., 2013). They showed that both sequence constraints (PhastCon scores) and evolutionary ages of candidate active enhancers peak at different developmental stages in different tissues. Though enhancers active in the brain show the highest conservation at E11.5, heart enhancers active at mouse E11.5 are less conserved compared to those active during earlier cardiac lineage specification (Figure 2A). This suggests that although enhancer turnover is a typical property of heart enhancers, deeply conserved CREs are more likely to be active in early cardiogenesis or even prior to cardiac lineage commitment.

To explore the existence of pre-cardiac enhancers that could contribute to the initiation of cardiac gene regulatory networks, we recently characterized the open chromatin landscape of a cardiac-enriched population in zebrafish embryos before the expression of the canonical cardiac marker nkx2.5 (Yuan et al., 2018). This approach allowed us to detect cardiac CREs that were primed early in development prior to cardiac lineage commitment. We present this work in Figure 2 as a general example of how comparative genomic resources in combination with epigenomic profiling in two or more species can give insight into functionally conserved developmental enhancers. To determine to what extent deeply conserved CREs were involved in early heart development we exploited conserved non-coding element (CNE) datasets established using both direct alignment and indirect approaches (Hiller et al., 2013; Braasch et al., 2016) and found more than 160 human-zebrafish conserved candidate heart enhancers (referred to as aCNEs). Though most of these aCNEs remain to be tested in vivo, the majority of the aCNEs tested (15/18) drive robust cardiac expression in zebrafish. This example illustrates a comparative strategy for discovering early heart enhancers underscores that at least some of the regulatory logic driving vertebrate heart development can be found in orthologous sequences shared between humans and fish.

In sum, despite the overall rapid evolution of heart enhancers, a small fraction of deeply conserved heart enhancers likely contributes to the regulation of early cardiogenesis. The lack...
Discovering conserved heart enhancers during early heart development: a case study. (A) Enhancers that are active at different stages of heart development show different evolutionary constraints. In mouse, enhancers that are active in mesoderm progenitors show higher sequence conservation than enhancers active in ESC and E11.5 embryonic hearts. But conservation levels of enhancers that active during the transition of mesoderm progenitors to cardiac progenitors and cardiac progenitors to cardiomyocytes remain less characterized. aCNEs, the accessible chromatin shared between zebrafish and human (or zebrafish and mouse) were identified within the mesoderm to cardiac progenitor transition (Yuan et al., 2018). Schematics generated based on Figure 5 (Nord et al., 2013). (B) Schematics showing sequence homology and shared enhancer signatures for aCNE1 locus across multiple species. aCNE1 was first discovered as an accessible chromatin region specific for an early cardiac progenitor-enriched population in zebrafish. Gray lines indicate the existence of orthologous sequences to aCNE1 in the given species (based on CNEs identified in Hiller et al., 2013). In mouse, aCNE1 regions are co-occupied by multiple cardiac TFs in cardiac cells (based on data from Luna-Zurita et al., 2016; Laurent et al., 2017). Human aCNE1 region shows chromatin accessibility in cardiac progenitor cells (based on data from Paige et al., 2012). The stickleback and the frog icons were created by Milton Tan and Soledad Miranda-Rottmann, respectively, and shared through http://phylopic.org/ under the following license (https://creativecommons.org/licenses/by/3.0/). (C) Genome browser view of aCNE1 in zebrafish (ZaCNE1) and human (HaCNE1) genome. aCNE1 is located 108 kb upstream of hand2 in the zebrafish genome and 406 kb upstream of HAND2 in the human genome. Yellow boxes highlight the genes flanking aCNE1, indicating the conserved synteny that aCNE1 resides in. ATAC-seq data from Yuan et al. (2018) is plotted for ZaCNE1 and promoter capture Hi-C data from Montefiori et al. (2018) is plotted for HaCNE1. Note that aCNE1 display conserved cardiac-specific activity in both zebrafish (accessibility) and human (interacting with cardiac gene HAND2). ZaCNE1 and HaCNE1 shares an aligned GATA motif, the mutation of which can be used to determine if the activity of aCNE1 depends on this GATA motif. (D) Functional enhancer assays of WT and GATA motif mutated zebrafish and human aCNE1 sequence in zebrafish embryos. Candidate sequences are cloned into an enhancer vector to drive GFP expression. The whole cassette will be chromatinized after injecting into zebrafish embryos. For both ZaCNE1 and HaCNE1, GATA motif mutation leads to decreased enhancer activity compared to the respective WT sequences. This example illustrates that human and zebrafish aCNE1 share conserved activity and regulation despite less than 60% sequence identity. Schematics generated based on data from Yuan et al. (2018). Parts of this figure were created with BioRender.com.
of overt sequence conservation in heart enhancers may be partially due to the rapid turnover of TFBSs. On the other hand, variants in heart enhancers that alter gene expression are likely to contribute to morphological differences of cardiac structures between species.

**Table 5** Chromatin accessibility datasets used for annotating heart enhancers.

| Methods          | Species          | Samples                          | Condition                                          | Stage                                      | References       |
|------------------|------------------|----------------------------------|----------------------------------------------------|--------------------------------------------|------------------|
| ATAC-seq         | Ciona            | B7.5 lineage                     | WT, Fgfr dominant-negative, Mek constitutively active, Foxf-CRISPR, M-Ras constitutively active | 10 hpf (multipotent cardiopharyngeal progenitors) | Racioppi et al., 2019 |
| ATAC-seq         | Mouse            | Heart                            | WT, gata5 -/-, hand2 -/-, tbx5 -/- mutants         | 72 hpf                                    | Pawlak et al., 2019 |
| ATAC-seq         | Mouse            | endocardial cells                | WT and TBX20 KO                                    | E12.5                                      | Zhou et al., 2017 |
| ATAC-seq         | Mouse            | Isl1+ cardiac progenitor cells   | WT                                                  | E7.5, E8.5, E9.5                           | Quaife-Ryan et al., 2017 |
| ATAC-seq         | Mouse            | Isl1+/CD31+, Isl1+/CD31- cardiac progenitor cells | WT                                                  | E8.5, E9.5                                | Jia et al., 2018 |
| ATAC-seq         | Mouse            | Isl1+ cardiac progenitor cells   | Nkx2.5 overexpression in Isl1+ cells               | E9.5, E12.5                               | Boogerd et al., 2017 |
| Omni-ATAC-seq    | Mouse            | Heart                            | WT                                                  | E8.5, E9.5                                | Liu et al., 2019 |
| ATAC-seq         | Mouse            | Ventricle cardiomyocytes         | WT                                                  | Adult                                      | Akerberg et al., 2019 |
| ATAC-seq         | Mouse            | Cardiac pacemaker cells (PCs), right atrial cardiomyocytes (RACMs) | WT                                                  | Neonatal (P0-P2)                          | Galang et al., 2020 |
| Single-cell ATAC-seq | Mouse      | Isl1+ cardiac progenitor cells                                                 | P1, P8 (3days post surgeries for both)              | Wang et al., 2020 |
| ATAC-seq         | Human            | ESCs, ESC-differentiated cells    | WT                                                  | ESCs, mid primitive streak, lateral mesoderm, cardiac mesoderm | Loh et al., 2016 |
| ATAC-seq         | Human            | iPSC-differentiated cells         | WT, GATA4_G296S                                     | iPS-derived cardiac progenitor cells       | Ang et al., 2016 |
| ATAC-seq         | Human            | ESCs, ESC-differentiated cells, iPSCs, iPSC-differentiated cells                | WT                                                  | ESCs and iPSCs, mesoderm, cardiac mesoderm | Liu et al., 2017 |
| ATAC-seq         | Human            | ESCs, ESC-differentiated cells, iPSCs, iPSC-differentiated cells                | Control and INN (isotretinoin) treatment            | ESCs and iPSCs, mesoderm, cardiac mesoderm | Liu et al., 2018 |
| ATAC-seq         | Human            | ESCs, ESC-differentiated cells    | WT                                                  | ESCs, mesoderm, cardiac progenitors, cardiomyocytes | Bertero et al., 2019 |
| ATAC-seq         | Human            | ESC-differentiated sinoatrial node-like pacemaker cells (SANLPC), ventricle-like cardiomyocytes (VLCM), | WT                                                  | ESC-differentiated cardiomyocytes          | van El et al., 2020 |

Data from large consortiums (ENCODE, FANTOM, and Roadmap Epigenomics Projects) are not listed. Datasets are sorted by species first and then by publication dates.

**Heart Enhancers: One Cell at a Time**

Currently, most of the data for annotating heart enhancers was generated at the bulk population level (Tables 3–5); however, both in vitro differentiated cardiac cells and animal hearts contain heterogeneous populations (reviewed in Paik et al., 2020). This
was largely due to the challenges in isolating closely related developmental lineages and collecting enough material from early embryos for enhancer profiling. But as enhancer activity is highly context-specific, the existing data bias likely limits the discoveries of enhancers that are active only in specific subpopulations (e.g., SHF progenitors, endocardial cells, cardiac smooth muscle cells, etc.) or at certain stages.

Rapid advances in single-cell genomics techniques have brought unprecedented opportunities to circumvent the difficulties in cell type isolation. Specifically, single-cell ATAC-seq (scATAC-seq) has become more and more commonly used in delineating cell-type-specific CREs within diverse cellular populations (Buenrostro et al., 2015; Cusanovich et al., 2015). scATAC-seq of Isl1+ cells from E8.5 and E9.5 mouse embryonic hearts revealed the TF regulators involved in the different stages of two distinct developmental trajectories, the cardiomyocyte and endothelial trajectories (Jia et al., 2018). More recently, scATAC-seq of neonatal hearts post-injury uncovered previously uncharacterized TFS that potentially regulate specific cell types in mammalian heart regeneration and decoded the cis and trans regulators underlying regenerative and non-regenerative injury responses (Wang et al., 2020). Moreover, large single-cell atlases of chromatin accessibility have been generated for 13 adult mouse organs (~100,000 nuclei) and 15 fetal human tissues (~800,000 nuclei), illustrating the regulatory programs that define the cell repertoire for many mammalian organs including the heart (Cusanovich et al., 2018a; Domcke et al., 2020). Embryonic single-cell accessible chromatin landscapes have been profiled for E8.25 mouse embryos (~19,000 nuclei) and Drosophila embryos (~20,000 nuclei) spanning early blastoderm to terminally differentiated lineages (Cusanovich et al., 2018b; Pijuan-Sala et al., 2020). As all the above studies provide a variety of processed data and interactive web sessions for convenient exploration of the chromatin accessibility of one’s favorite genes or loci, they can be very useful resources for exploring cell type-specific cardiac enhancers.

Furthermore, with single-cell multimodal omics being selected as the Methods of the Year 2019 (Nature Methods, 2020), techniques for simultaneous measuring multiple modalities in the same single cells are blooming rapidly. Related to epigenomics, it has become possible to simultaneous profile accessible chromatin and transcriptome (Cao et al., 2018; Chen et al., 2019; Li et al., 2019; Moudgil et al., 2020), methylome and transcriptome (Angermueller et al., 2016), methylome and chromatin conformation (Li et al., 2019), or even three modalities altogether (Pott, 2017; Clark et al., 2018) within the same cells. The combinatorial use of single-cell epigenomics techniques on cardiac samples will potentially provide a holistic view of enhancer activities in all subtypes of cardiac cells across all stages in heart development. The multi-omics measurements not only enable a more comprehensive and accurate delineation of the state of the single cells but also provide unique opportunities in identifying the potential causal factors across multiple regulatory layers, by correlating changes from genetic, epigenetic, or chromatin conformation levels to the gene expression differences. Although technology and analytic challenges still lie ahead, the application of single-cell epigenomics, especially the multi-omics approaches, into heart development, will likely transform the way that we study and understand heart enhancers and cardiac gene regulatory networks.

**Computing Heart Enhancers**

With the rapid accumulation of hundreds of epigenomic and transcriptomic datasets from cardiac tissues, efforts have been made toward compiling them and extract sequence features from known cardiac enhancers to predict unknown ones. Dickel et al. (2016) conducted an integrative analysis of over 35 genome-wide H3K27ac or P300 profiles from mouse or human heart samples to compile a compendium of more than 80,000 heart enhancers, which serves as one of the most comprehensive putative heart enhancer lists available to date. The abundance of genomics datasets and the growing number of in vivo validated heart enhancers also provide ample input for building computational models for novel heart enhancer prediction. One kind of model is purely based on the sequence features of the gold standard heart enhancer experimentally validated in vivo. For example, Narlikar et al. (2010) combined motif discovery, Markov sequence feature characterization, and linear regression to build a heart enhancer classifier from ~70 validated heart enhancers. They used this classifier to discover more than 40,000 putative heart enhancers within the conserved CNEs in the human genome, with an in vivo validation rate > 60% (Narlikar et al., 2010). By comparing validated cardiac and non-cardiac enhancer sequences from Drosophila, Jin et al. (2013) identified a novel motif as a classifier for heart enhancer prediction. They further showed that this motif was essential for driving cardiac activity in 3/8 enhancers tested. One widely used sequence-based machine learning method, gapped k-mer support-vector-machine (gkm-SVM) (Ghandi et al., 2014), has been applied to learn the sequence features from previously identified open chromatin regions. It predicted an addition of 80,000 putative cardiac CREs and the cognate TFS that bind to them (Lee et al., 2018).

Several studies have explored how including different genomics features in training models could affect their performance in enhancer prediction. A study in Drosophila added ChIP signals on top of sequence motifs into their classifiers and found this combined strategy significantly boosted the prediction accuracy of cell-type-specific cardiac enhancers than motif sequence alone (Ahmad et al., 2014). By further including ChIP data for a larger set of cardiac TFs and histone modifications, their updated model was able to distinguish enhancers active in distinct subpopulations of cardiac cells and pericardial cells in Drosophila embryos (Busser et al., 2015). Similarly, Akerberg et al. (2019) took advantage of the variety of ChIP-seq data that they generated for mouse hearts and compared the performance of different chromatin features (open chromatin, H3K27ac histone modification, cardiac TF occupancy) alone or combined in predicting heart enhancers. They found open chromatin had high sensitivity while TF binding profiles yielded high precision in enhancer prediction. Ultimately, the number of co-bound cardiac TFs turned out to be the most important classifier in heart enhancer prediction compared to signal intensities (Akerberg et al., 2019).
With the rapid evolution of the machine learning field, computational classification and predictions will become an important component that is complementary to experimental data in heart enhancer characterization. The two strategies will benefit from the advancement of each other and together expand our understanding of enhancer biology.

HEART ENHANCERS IN CARDIOVASCULAR DISEASE

Heart diseases are a leading cause of death worldwide (Mozaffarian et al., 2015). As the most prevalent human birth defects, congenital heart disease (CHD) affects roughly 0.8% of newborns (Fahed et al., 2013). Though disruption of a set of developmental and structural genes have been recognized as the causes of a portion of CHD, the genetic factors underlying a large number of cases remain ambiguous (Fahed et al., 2013; Barnett and Postma, 2015; Postma et al., 2015; Richter et al., 2020). Genome-wide association studies (GWAS) have been carried out to identify the underlying genetic causes of a wide range of cardiovascular phenotypes and diseases, including CHD, cardiac arrest, coronary artery disease (CAD), cardiac arrhythmia, cardiomyopathy, and myocardial infarction (Arking et al., 2014; Nikpay et al., 2015; Eppinga et al., 2016; Nelson et al., 2017). Currently, thousands of variants have been implicated in heart-related disease risks (NHGRI GWAS catalog 1).

Whole-genome sequencing (WGS) is becoming the method of choice for discovering de novo variants in CHD. Supporting the use of WGS for discovering molecular mechanisms underlying CHD, a recent study illustrated that the potential contribution from disruptive non-coding variants was at least as high as that from coding-variants (Richter et al., 2020). However, several factors complicate the functional annotation of disease-associated non-coding variants (Zhang and Lupski, 2015). In the case of common genetic variation associated with CHD-related phenotypes uncovered by GWAS, the tagged SNPs used will be in linkage disequilibrium (LD) with other SNPs that may represent the true causal variant. Even if a likely pathogenic non-coding mutation or copy number variation is nominated, one must then ascertain when and where this change impacts development and disease. In the following section, we briefly review insights into heart enhancer function revealed by human genetic studies.

Connecting Non-coding Variants to Cardiovascular Diseases

Only a handful of non-coding variants linked to cardiovascular diseases have been functionally dissected (Table 6). Compared to studying the function of a protein coding gene mutation, the functional characterization of non-coding disease associated variants is challenging. An early example of this was done for a genetic variant on human chromosome 9p21 harboring multiple SNPs associated with myocardial infarction and CAD (reviewed by Samani and Schunkert, 2008). A large 70 kb deletion of the whole orthologous sequence in the mouse genome severely reduced the expression of the nearby cardiac genes (Cdkn2a/b) and affected aortic smooth muscle cell proliferation and senescence. Allele-specific analysis of Cdkn2b transcripts in the heterozygous mice revealed a lack of cis-acting enhancers as the main mechanism underlying Cdkn2b downregulation, suggesting this genetic susceptibility interval contains enhancers that could be affected by the discovered sequence polymorphisms (Visel et al., 2010). However, disruption of cis-regulatory elements is not the only mechanism that contributes to diseases risk. Other studies revealed that expression of the long non-coding RNA (lncRNA) ANRIL, which resides in chromosome 9q21, was affected by several SNPs within this region, and ANRIL, in turn, could regulate other genes involved in vascular cell proliferation, adhesion, apoptosis, and remodeling (Holdt et al., 2010; Congrains et al., 2012a,b).

Another well-studied example is rs12190287, a CAD-associated variant located within the 3' UTR of the TCF21 gene. Two continuous studies together revealed a dual mechanism of this SNP in modulating TCF21 expression at both transcriptional and post-transcriptional levels (Miller et al., 2013, 2014). Overlapping a TCF21 enhancer, this variant causes dysregulation of TCF21 through allele-specific histone modifications (H3K4me1, H3K27ac, H3K27me1) and AP-1 factor (c-Jun, JunD, ATF3) binding. These allele-specific chromatin effects are further augmented upon PDGFR-β stimulation, which indicates that the vascular growth factor signaling also acts differently on this variant (Miller et al., 2013). Moreover, the same minor allele disrupts a miR-224 binding site within the 3' UTR of TCF21, therefore, prevents the post-transcriptional repression of TCF21 mediated by this miRNA (Miller et al., 2014).

The ion channel genes SCN5A/SCN10A locus is another hotspot heavily loaded with variants linked to cardiac arrhythmia and conduction system disorders (Veerman et al., 2015). One cardiac arrhythmia-associated SNP rs6801957 is located within the intron of SCN10A but is encompassed by a human-mouse conserved enhancer that interacts with the nearby gene SCN5A (van den Boogaard et al., 2014). This variant, but not other variants in LD disrupts the binding of TBX3/TBX5 in vitro and reduces the activity of this enhancer in the cardiac conduct system (van den Boogaard et al., 2012). Overall, these variant-oriented studies revealed the molecular mechanisms through which single nucleotide substitutions could alter enhancer activity and lead to pathological gene expression.

Discovering Disruptive Non-coding Variants Near Cardiac Genes

The CREs controlling the expression of TFs (i.e., the regulators of the regulators) are prime candidate regions for discovering damaging mutations that lead to gene dosage-related phenotypes (van der Lee et al., 2020). Indeed, hypothesis driven dissection of enhancers near cardiac genes have revealed several examples of disease causing non-coding mutations that control haploinsufficient cardiac genes TBX5, NKX2.5, and SHOX2 (reviewed in Chung and Rajakumar, 2016; Steimle and Moskowitz, 2017; Li et al., 2018).
It had been known for over a decade that heterozygous mutations within TBX5 lead to Holt-Oram syndrome in humans (Basson et al., 1997, 1999) when Smemo et al. (2012) went searching for disease-causing enhancer mutations around the TBX5 gene in families with septal defects, the predominant cardiac defect of Holt-Oram syndrome. This study, which involved scanning more than 700 kb for conserved non-coding sequences revealed three enhancer elements which together recapitulated the endogenous TBX5 heart expression in developing mouse embryos. Targeted sequencing revealed homozygous mutations in one of the enhancer elements in individuals with, but not in family members without, the disease. Another targeted sequencing of the NKX2.5 locus in ventricular septal defect patients revealed novel variants within the NKX2.5 promoter and a known distal enhancer (AR1). These novel variants significantly altered the transcriptional activity of the Nkx2.5 promoter and AR1 enhancer in luciferase assays (Pang et al., 2012; Huang et al., 2013). These tour de force experiments illustrate the lengths one must go to implicate regulatory mutations as a disease causing mechanism, and demonstrates how understanding the molecular mechanisms underlying human disease can reveal fundamental biological insights in cardiac enhancer elements.

In addition to enhancers and promoters, non-coding regulatory variation can impact miRNA binding sites, lncRNAs, or even several of these functional elements at the same time. In principle this could occur by disrupting or creating TF/miRNA binding sites, changing chromatin states, mediating different responses to extracellular signaling, or affecting lncRNA expression which in turn can affect gene regulation in trans (Table 6). For example a variant associated with increased CHD susceptibility was identified within the 3′ UTR of TBX5. This variant was shown to increase the binding of two miRNAs with the minor allele leading to a significant reduction in the expression of TBX5 through transcriptional and translational regulation (Wang et al., 2017). NKX2.5 mutations have also been implicated in diverse types of CHD, including ventricular septal defects (reviewed in Chung and Rajakumar, 2016). Similarly, target sequencing of the SHOX2 region in atrial fibrillation (AF) patients identified an AF-associated SNP within the 3′ UTR. The 3′ UTR allele created a binding site for an mRNA miR-92b-5p, which significantly reduced the SHOX2 3′UTR reporter activity in a luciferase assay (Hoffmann et al., 2016).

While there are relatively few hard-won examples of non-coding mutations that explain the molecular mechanism behind CHD, it is clear that a comprehensive annotation of heart enhancer location and function will accelerate molecular-based diagnoses and our understand of heart gene regulation.

Interpreting Non-coding Variants With Genome-Wide Enhancer Annotation

With the burst of cardiac epigenomic datasets in the past decade, the interpretation of heart disease-associated variants has developed from susceptible locus-centric to a genome-wide manner. Continuous efforts have been made to first establish a comprehensive enhancer annotation and then use for the fine-mapping non-coding variants (Dickel et al., 2016; Choy et al., 2018; Montefiori et al., 2018). For example, the heart enhancer list that they curated from ChIP-seq datasets, Dickel et al. (2016) found more than 2000 enhancer-overlapping variants that were associated with heart phenotypes. When deleting two of the variant-containing enhancers that were upstream of cardiac structure genes (Myl7 and Myl2), they showed that both enhancers are required for normal cardiac gene expression, cardiomyocyte morphology, and heart functions. On top of enhancer identification, chromatin conformation capture assays are especially helpful for linking cardiac GWAS SNPs to their targeted genes. The promoter capture Hi-C datasets generated in differentiated cardiomyocytes arguably pinpoint the true target genes of many GWAS and LD SNPs, some of which were different from the target genes proposed based on proximity (Choy et al., 2018; Montefiori et al., 2018). Remarkably, Montefiori et al. (2018) reported that 90% of the SNP-gene interactions skipped at least one gene promoter, arguing against the intuitive approach of assigning SNPs to their neighboring genes when interpreting possible causal mechanisms. In line with the cell-type-specificity of enhancer activities, the interaction networks identified using cardiomyocyte promoter capture Hi-C data turned out to be most informative to interpret cardiac arrhythmia phenotypes (which directly results from cardiacmyocyte dysfunction) as compared to CHD, CAD, heart failure, and myocardial infarction (all of which involved cellular systems other than cardiomyocytes) (Choy et al., 2018; Montefiori et al., 2018). This indicates that generating chromatin maps for other cardiac cell types or at other differentiation stages could more effectively facilitate the mechanistic dissection of other types of cardiovascular diseases.

With the promising future of functional genomics in non-coding variants dissection, generation and curation of transcriptome and epigenome datasets have been tailed toward studying a specific type of heart disease to achieve higher precision. For example, to understand causal variants for atrial fibrillation (AF), RNA-seq data and ATAC-seq specifically from the left atria were generated to identify potential CREs and target genes that were likely to be affected by the genetic variants within 104 AF-associated loci (van Ooijerkerk et al., 2019). Following this study, a functional enhancer screening of these AF-associated loci using STARR-seq found 24/55 the variant-containing enhancers with allele-specific activities, demonstrating the robustness of this approach. Deletion of the orthologous region of one such enhancer near Hcn4 in the mouse genome caused a loss of Hcn4 expression and cardiac defects (van Ooijerkerk et al., 2020).

In addition to our growing understanding of the regulatory logic underlying developmental gene expression, it is also important to acknowledge the contribution of pro-inflammatory processes on heart enhancer usage and gene expression. For instance, the rapid pro-inflammatory gene expression by the NF-κB transcription factor complex, which across cell types utilizes clusters of strong enhancers (also known as “super enhancers”) to rapidly deploy pro-inflammatory gene expression (Brown et al., 2014; Schmidt et al., 2015).
TABLE 6 | Functionally characterized non-coding SNPs implicated in cardiovascular disease.

| SNP       | SNP position | Gene(s)          | Disease                      | Evidence                                                                 | References                  |
|-----------|--------------|------------------|------------------------------|--------------------------------------------------------------------------|-----------------------------|
| rs6801957 | chr 9p21     | Cdkn2a/b         | coronary artery disease      | Deletion of the mouse orthologous interval severely impacts Cdkn2a/b expression nearby through a cis-acting mechanism. | Visel et al., 2010          |
| rs1190128 | 3' UTR of TCF21 | TCF21            | coronary heart disease      | The protective allele disrupts AP-1 binding and enhancer-associated histone modification, leading to TCF21 expression changes. | Miller et al., 2013         |
| rs897612 | 3' UTR of TCF21 | TCF21            | coronary heart disease      | The protective allele (G) changes TCF21 transcript structure and disrupts miR-224 binding and post-transcriptional repression mediated by this miRNA. TGF-b and PDGF-bb signaling act upstream of miR-224 mediated allele-specific expression. | Miller et al., 2014         |
| rs11290287 | G>A, overlaps an enhancer | Intron of SCN10A | SCN5A | The enhancer interacts with the SCN5A promoter. The minor allele disrupts a T-box binding site and impairs the enhancer activity in the cardiac conduction system. | van den Boogaard et al., 2012, 2014 |
| rs7539120 | An upstream enhancer of NOS1AP | NOS1AP | QT interval variations | The risk allele leads to increased enhancer activity. Overexpression of NOS1AP result in altered electrophysiology in cardiomyocytes | Kapoor et al., 2014         |
| rs4897612 | 137' in VNN1 promoter | VNN1 | HDL cholesterol levels | eQTL of VNN1, allele-specific transcriptional activity, chromatin accessibility, binding of nuclear protein including SP-1, methylcylation and chromatin condensation | Kaskow et al., 2014         |
| rs2050153 | −587' in VNN1 promoter | VNN1 | HDL cholesterol levels | eQTL of VNN1, allele-specific chromatin accessibility, methylcylation and chromatin condensation | Hoffmann et al., 2016       |
| rs139912749 | T>C, overlaps a miRNA binding site | 3' UTR of SHOX2 | atrial fibrillation | The minor allele creates a functional binding site for miR-92b-5p, which leads to reduced expression of SHOX2. | Wang et al., 2017           |
| rs8489566 | 3' UTR of TBX5 | TBX5             | CHD susceptibility          | The minor allele shows increased binding to miR-9-30a, which leads to reduced expression of TBX5 | Kapoor et al., 2019         |
| rs7373779, rs41312411, rs11710077, rs13097780, rs6801957 | SCN5A-SCN10A GWAS locus | SCN5A | QT interval variations | Allele-specific enhancer activity and nuclear factor binding. (More putative variants were identified other than these five representative ones) | Visel et al., 2016          |

This mode of gene regulation can recruit transcriptional machinery from cell-lineage genes in a process known as cofactor squelching (Schmidt et al., 2015, 2016). Indeed a detailed knowledge of acute and chronic inflammatory enhancer biology during heart development and disease is essential and integrating this information with emerging compendiums of heart epigenomic data (such as Vanoudenhove et al., 2020) will be valuable.

Integrating enhancer information into the functional annotation of non-coding variants is no doubt a powerful approach; however, it should be noted that disrupting enhancer activities is not the only mechanism underlying the pathological consequences of non-coding variants. Even with extensive efforts in curating heart enhancers, nearly 90% of the heart disease-associated LD SNPs did not overlap any heart enhancers in the compendium (Dickel et al., 2016) and more than 80% of them could not be linked to gene promoters based on cardiomyocytes promoter capture Hi-C data (Montefiori et al., 2018). Apart from other possible technical reasons, this small overlap suggests regulatory mechanisms other than altering heart enhancers could account for a substantial portion of non-coding variants-mediated disease risk. In fact, unbiased examination of 98 amplicons (250–600 bp) containing 106 SNPs linked to QT interval phenotypes at the SCN5A locus found that 35% of the reference allele-containing amplicons showed enhancer activity while another 44% worked as silencers. Besides CREs, functional non-coding variants have also been mapped to miRNA-binding sites and lncRNAs (Table 6). A recent CHD genomic analysis has demonstrated significant enrichment of RNA-binding-protein regulatory sites in de novo variants identified in CHD patients, indicating contribution from disrupted post-transcriptional regulation to CHD (Richter et al., 2020). Moreover, it has been shown that the same minor allele of a variant could regulate the
target gene expression through both transcriptional and post-transcriptional mechanisms and, even more strikingly, in an opposite manner, highlighting the complexity of sequence polymorphisms in affecting gene expression (Miller et al., 2013, 2014). Therefore, a comprehensive annotation of different types of cardiac CREs that are not limited to enhancers, together with a good non-coding RNA annotation, will be necessary for truly understanding the mechanisms of the heart disease from the non-coding variant perspective. Additionally, it is likely that several coding and/or non-coding variants collectively explain a complex cardiovascular phenotype. Thus while it is important to dissect disease phenotype associated variants individually, more complex studies looking at genetic interactions and addictive effects may well be required.

EMERGING TECHNIQUES FOR THE FUNCTIONAL DISSECTION OF HEART ENHANCERS

So far, numerous putative heart enhancers have been identified in different conditions and cell types from several model organisms. However, compared to enhancer mapping, the throughput of current approaches for enhancer functional dissection, especially in vivo, remains a major bottleneck. Traditionally, each candidate enhancer is accessed individually via being placed upstream of a reporter gene and introduced into cells or in vivo organisms. Collective efforts using this approach have led to the establishment of central resources of validated enhancers, such as the Vista Enhancer Browser2 (Visel et al., 2007). To measure enhancer activity in a more high throughput manner, several methods have been developed through the years, such as massively parallel reporter assays (MPRA) (Melnikov et al., 2012; Patwardhan et al., 2012; Sharon et al., 2012), and self-transcribing active regulatory region sequencing (STARR-seq) (Arnold et al., 2013). However, most of these approaches are typically carried out in vitro or in the absence of chromatin contexts, raising the question of how faithfully their results reflect the native activities of the candidate regions. Recently, the development of more robust and scalable in vivo enhancer assays, such as the site-directed enhancer-reporter assay (enSERT), has allowed systematic assessment of more than 100 variants in an essential limb enhancer (Kvon et al., 2020). For invertebrates like Drosophila, unbiased, automated enhancer mutational scanning has been established using robotic systems, which permits multi-stage quantitative measurement of enhancer activities in development (Fuquai et al., 2020). Developing similar systems for vertebrates will greatly improve our capacity in assessing vertebrate enhancer functions and advance our understanding of how regulatory information is encoded in developmental enhancers.

2https://enhancer.lbl.gov/

Compared to all enhancer reporter assays, which introduces an atypical distance between candidate enhancers and the reporter genes, a complementary perhaps preferred way to understand enhancer functions is to dissect their activity and function in their endogenous loci. The ever-growing CRISPR-Cas9 toolbox provides many options for in situ enhancer dissection (reviewed in Klein et al., 2018; Pickar-Oliver and Gersbach, 2019; Xu and Qi, 2019). Individual enhancer deletions or substitutions have been routinely used to characterize enhancer functions in specific developmental processes (Dickel et al., 2016, 2018; Kvon et al., 2016; Osterwalder et al., 2018; van Elf et al., 2020; van Ouwerkerk et al., 2020). To increase the throughput, a variety of CRISPR-based enhancer screens have been developed for in vitro systems, such as the saturated tilling arrays that can unbiased assess certain genomic loci for functional enhancers (Korkmaz et al., 2016; Diao et al., 2017; Gasperini et al., 2017) and epigenetic screens against candidate enhancers using deactivated Cas9 (dCas9) coupled with transcriptional activators or repressors (Klann et al., 2017; Simeonov et al., 2017; Fulco et al., 2019; Gasperini et al., 2019). Specifically, by using single-cell RNA-seq as readouts, CRISPR-mediated epigenetic screens have been successfully applied to perturb thousands of candidate enhancers in cell lines to determine their functional importance and target genes (Fulco et al., 2019; Gasperini et al., 2019). Though achieving the same throughput in vivo may still be challenging, increasing efforts have been made toward applying these powerful systems in animals. Very recently, a single-cell-based in vivo CRISPR/Cas9 screen (Perturb-seq) has been successfully used to screen 35 genes in the mouse developing neuronal cortex in utero (Jin et al., 2020). Though not large-scale yet, this study offers a very encouraging framework to achieve systematic assessment of genes or CREs in vivo. Moreover, dCas9-mediated epigenetic perturbation, which is likely more suitable for enhancer screens, has been continuously optimized over the years and showed a promising future of targeting enhancers in a more scalable manner in developing animals (Morita et al., 2016; Zhou et al., 2018; Li et al., 2020).

DISCUSSION, CONCLUDING REMARKS, AND FUTURE PERSPECTIVES

The past decade has witnessed an exponential growth of the numbers of putative heart enhancer regions identified, largely owing to rapid advances in epigenomic profiling approaches. These techniques are still growing at an ever-increasing speed and will undoubtedly continue to revolutionize the way that researchers annotate and interpret enhancer activities. Single-cell epigenomic techniques, especially the multi-omics approaches, will likely become one of the main driving forces in expanding the horizon of cardiac enhancers and regulatory networks in the next decade. However, it should be noted that many analytical challenges are inherently associated with single-cell epigenomic datasets that currently remain sparse and noisy (reviewed
until we can conduct mid- to large-scale dissections in the near future. With the concurrent advancement to make a substantial contribution to functional enhancer use of CRISPR technologies and single-cell genomics is likely opportunities to overcome these challenges. The combined is required.

specific contexts in which a given developmental enhancer therefore becomes a very complicated task to determine the et al., 2010; Perry et al., 2010; Osterwalder et al., 2018). It stressed environments or sensitized genetic backgrounds (e.g., such as heterozygous deletion of developmental TFs) (Frankel et al., 2010; Perry et al., 2010; Osterwalder et al., 2018). While these redundant enhancers may be seemingly dispensable in normal conditions, they could be required in stressed environments or sensitized genetic backgrounds (e.g., such as heterozygous deletion of developmental TFs) (Frankel et al., 2010; Perry et al., 2010; Osterwalder et al., 2018). It therefore becomes a very complicated task to determine the specific contexts in which a given developmental enhancer is required.

On the other hand, we are in an era with unprecedented opportunities to overcome these challenges. The combined use of CRISPR technologies and single-cell genomics is likely to make a substantial contribution to functional enhancer dissections in the near future. With the concurrent advancement of these two technologies, it probably will not be too far until we can conduct mid- to large-scale in vivo enhancer screening. Moreover, coupling CRISPR with other single-cell epigenomic assays (e.g., single-cell accessibility chromatin) to target TFs or chromatin modifiers (Rubin et al., 2019; Sanjana et al., 2020), can provide information complementary to enhancer screens and together build toward a comprehensive regulatory network.

From traditional approaches to the newest genomic assays, the rich history of heart enhancer studies has not only led us with a wealth of knowledge about the genomic locations, functional roles, evolutionary conservation, and disease implications of heart enhancers but also opened up many challenges and unanswered questions. What are the best experimental designs and analytic strategies of single-cell epigenomic assays? How can we increase the scalability of functional enhancer assays and efficiently adopt them into in vivo contexts? Could we develop more robust and transferable computational methods that can not only predict heart enhancers but also determine their chamber-, cell-type or developmental-stage specific activities and how the activity of enhancers can be affected by non-coding variants? We may not be sure when these questions will be fully answered, but we can confidently anticipate that efforts made in tackling these challenges will push our understanding of heart enhancers and cardiac regulatory network to an unprecedented level.

AUTHOR CONTRIBUTIONS

XY researched, conceived the structure, created the figures and tables, and led the writing of the review. MW and IS developed the ideas and provided text for the review. All the authors read and edited the review.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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