Tissue-specific H2Bub1 is required for cardiomyocyte development

Syndi Barish

Jeffrey Drozd

Yale University School of Medicine

Kathryn Berg

Yale University School of Medicine

Isabella Berglund-Brown

Yale University School of Medicine

Labeeqa Khizir

Yale University School of Medicine

Lauren Wasson

Harvard Medical School <https://orcid.org/0000-0002-5193-5215>

Sidi Chen

Yale University <https://orcid.org/0000-0002-3819-5005>

Martina Brueckner (✉ martina.brueckner@yale.edu)

Yale University School of Medicine <https://orcid.org/0000-0003-0347-5389>

Article

Keywords: H2Bub1, congenital heart disease, tissue-specific regulation, RNF20-core complex

Posted Date: August 24th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-788134/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Tissue-specific H2Bub1 is required for cardiomyocyte development

Syndi Barish¹, Jeffrey Drozd², Kathryn Berg¹, Isabella Berglund-Brown², Labeeqa Khizir²,
Lauren K Wasson^{3,4}, Sidi Chen¹, Martina Brueckner^{1,2,*}

Departments of Genetics¹ and Pediatrics²
Yale University School of Medicine,
333 Cedar Street,
New Haven, Connecticut 06510, USA

Department of Genetics³,
Harvard Medical School
Boston, MA 02115, USA

Howard Hughes Medical Institute, Division of Cardiovascular Medicine⁴
Brigham and Women's Hospital
Boston, MA 02115, USA

*Correspondence to:
Martina.brueckner@yale.edu

Abstract

De-novo mutations affecting Histone2B-K120 monoubiquitination (H2Bub1) are enriched in congenital heart disease; however, how H2Bub1 affects heart morphogenesis beyond its function in establishing left-right asymmetry remains unknown. Here we show that the RNF20-core complex (RNF20-RNF40-UBE2B), which catalyzes H2Bub1, is required for cardiac development *in-vivo* in mice and *in-vitro* in iPSC-derived cardiomyocytes. Mice with cardiac-specific deletion of *Rnf20* have abnormal myocardium and ventricular septal defects; iPSCs with mutations affecting H2Bub1 cannot efficiently differentiate into cardiomyocytes. Sarcomeric gene expression is dependent on normal H2Bub1 both in mice and in iPSC-derived cardiomyocytes. Finally, we identify an accumulation of H2Bub1 near the center of tissue-specific genes in cardiomyocytes and MEFs associated with transcriptional efficiency that is reduced in *UBE2B*^{-/-} cardiomyocytes. In summary, normal H2Bub1 distribution is required in cardiac development, and H2Bub1 accumulation acts as a general mechanism for tissue-specific regulation of transcriptional elongation efficiency.

Introduction

Congenital heart disease (CHD), a structural abnormality of the heart and/or great vessels, is the most common cause of mortality from congenital malformations. Although 90% of patients survive to adulthood, many suffer from co-morbidities including myocardial dysfunction and neurodevelopmental abnormalities¹. Whole-exome sequencing of CHD patients identified variants in a broad spectrum of chromatin modifier genes in 2.3% of cases (including H3K4 and H3K37 methylation and H2BK120 monoubiquitination)²⁻⁴. Chromatin modifiers have previously been linked to heart development through animal models. Knock-out models of chromatin modifiers, such as *Kmt2d* (H3K4 methyltransferase), *Dot1l* (H3K79 methyltransferase), and *Hdac1/Hdac2* (histone deacetylases), result in a range of congenital heart abnormalities in vertebrates⁵⁻⁸. Further, many chromatin marks, including H3K4me3 and H3K27me3, are dynamic throughout cardiac lineage commitment. The correlation of chromatin patterns with stage-specific gene expression suggests that normal regulation of chromatin modifications is important for cardiomyocyte development⁹.

Although several chromatin modifiers have been directly linked to heart development, how monoubiquitination of histone H2B on K120 (H2Bub1) affects cardiogenesis is largely unknown. The H2Bub1 machinery was first discovered in yeast; H2B is monoubiquitinated by Bre1 (ubiquitin ligase), Rad6 (ubiquitin-conjugating enzyme), and Lge1 (scaffolding protein)¹⁰⁻¹². In mammals, the E3 ubiquitin ligases RNF20 and RNF40 correspond to Bre1, the Ubiquitin Conjugating enzyme E2 B (UBE2B) corresponds to Rad6, and the WW Domain-containing adaptor with coiled-coil (WAC) corresponds to Lge1¹³⁻¹⁶. Unlike most histone marks, H2Bub1 is located on gene bodies, where it is enriched near the promoter and gradually decreases towards the 3' end¹⁷. In order for the complex to add H2Bub1 *in-vitro*, Bre1 and Rad6 form a catalytic

shell around Lge1 enabling a liquid-liquid phase separation, which then recruits the nucleosome. If the ratios of the complex components change, so does its efficiency¹⁶. Thus, it is hypothesized that *in-vivo* in mammals, the ratios of the components affect the efficiency of the complex, and thus differential component expression can dictate where H2Bub1 is added.

CHD patients show an enrichment in *de novo* mutations affecting the RNF20 interactome, compared to controls, demonstrating a clinical relevance of H2Bub1 in CHD. Presently, the only known cardiac roles of H2Bub1 are in regulating cardiac situs by controlling cilia gene expression at the left-right organizer and in post-natal cardiomyocyte maturation^{18,19}. However, H2Bub1 has broad biological functions including differentiation, tumor suppression, and inflammation²⁰⁻²⁶. H2Bub1 is likely an activating mark, since it interacts with the Set1/COMPASS methyltransferase complex, which in most chromatin contexts creates activating marks²⁷. However, in *Rnf40* knockouts, there are genes near regions that lost H2Bub1 and also have increased expression, suggesting H2Bub1 can also be repressive²⁸. Further studies suggest that gene activation requires both the addition and removal of H2Bub1²⁹. These conflicting results indicate that the effect of H2Bub1 on transcriptional regulation is still not well understood.

H2Bub1 is deposited co-transcriptionally and co-localizes with RNA Pol II. As a result, H2Bub1 is highly correlated with transcriptional elongation, hinting at a positive regulatory mechanism³⁰. In yeast, the Paf1 complex (positive regulator of transcriptional elongation) has a direct and specific interaction with Bre1³¹. H2Bub1 also positively regulates the histone chaperone FACT, which promotes transcriptional elongation³². Conversely, RNF20 inhibits recruitment of TFIIS (which prevents RNA Pol II from stalling), effectively blocking transcriptional elongation²³. This suggests that H2Bub1 may also negatively affect

transcriptional elongation. Interestingly, increased H2Bub1 levels, due to mutations affecting deubiquitination, result in embryonic lethality in mice, but do not affect global Pol II transcription elongation in mouse embryonic stem cells (mESCs) or mouse embryonic fibroblasts (MEFs)³³. Thus, as with transcriptional regulation, the role of H2Bub1 on transcriptional elongation may be context-dependent.

Here we present analysis on the role of H2Bub1 in cardiac morphogenesis beyond its known role in establishing left-right asymmetry, both *in-vivo* in mouse and *in-vitro* in human iPSC-derived cardiomyocytes. Deletion of *Rnf20* in mouse hearts leads to embryonic lethality, abnormal compact myocardium, disorganized cardiac sarcomeres lacking an H zone, and ventricular septal defects. We further show that the stoichiometric ratios of the RNF20-complex components affect the deposition of H2Bub1, which limits cardiomyocyte differentiation *in-vitro*. H2Bub1 is abundant in iPSCs before becoming selectively maintained on a subset of genes significantly enriched in sarcomeric calcium genes during the transition from cardiac mesoderm to cardiac progenitor; this same set of genes is misregulated when H2Bub1 is decreased *in-vitro* and *in-vivo*. Finally, we establish the importance of H2Bub1 localization within a subset of genes notable for tissue-specific expression. We show that the location of H2Bub1 on these long genes is correlated with their transcriptional efficiency. Together, our data indicate that H2Bub1 is essential for cardiac development through regulation of cardiac sarcomeric genes during cardiomyocyte differentiation and development.

Results

***Rnf20* is required for mouse development**

Since *RNF20* variants have been linked to human CHD both with and without abnormal laterality, we asked how loss of RNF20 affects cardiogenesis in mice. Analysis of RNF20 protein in mouse embryonic hearts demonstrated ubiquitous cardiac expression at e9.5 (**Fig. S1a**) that becomes gradually restricted to only the epicardium at birth (**Fig. S1b**). Myocardial RNF20 forms an expression gradient with higher RNF20 at the myocardial surface compared to the lumen, and disappears by e16.5 (**Fig. S1c**). Endocardial RNF20 also disappears by e16.5 (**Fig. S1d**). The human CHD-associated mutation in *RNF20* is an early stop codon, suggesting haploinsufficiency for *RNF20*, so we analyzed constitutively heterozygous *Rnf20* mice containing a targeted deletion of *Rnf20* by replacing all coding exons with a LacZ reporter (obtained from KOMP2) (**Fig. S2a**)^{18,34}. The *Rnf20*^{+/-} mice are phenotypically normal and survive to adulthood with no discernible cardiac abnormalities (**Fig. S2b**). However, no *Rnf20*^{-/-} embryos are recovered post-blastocyst stage, consistent with previous data showing RNF20 is required for preimplantation development (**Fig. 1a, S2b**)²⁵.

To test whether RNF20 is required in cardiac development, we generated mice with cardiac-specific deletions of *Rnf20* using a conditional *Rnf20*^{fl} allele containing loxP sites flanking exons 2-4 of the *Rnf20* gene³⁵. Since the heart is formed by two waves of cell migration (first-heart field (FHF) predominantly contributing to the atria and left ventricle, and second-heart field (SHF) predominantly contributing to the outflow tract), we made FHF and SHF specific *Rnf20* deletion mouse lines using *Nkx2.5Cre* (FHF) and *Isl1Cre* (SHF) drivers (**Fig. S2c**)^{36,37}. We evaluated timing and specificity of Cre expression by mating with ROSA^{mt/mg} and evaluating Cre expression (**Fig. 1a**). *Rnf20*^{fl/-}::*Isl1Cre*⁺ mice survive to at least 7 months at

Mendelian ratios (**Fig. 1a, S2d**). They also have a normal heart/body weight ratio and hearts appear morphologically normal by H&E staining (**Fig. S2e, S2f**).

NKX2.5 is a transcription factor initially expressed in the entire heart, and then becomes restricted to the FHF³⁶. *Rnf20*^{fl/-}::*Nkx2.5*Cre⁺ embryos are found at Mendelian ratios until e12.25, but none were recovered after e12.5 (**Fig. 1a, S2h**). At e11.5, the embryos and heart appear normal by H&E, including 100% normal heart looping direction (**Fig. S2g**). By e12.25, the compact myocardium is significantly thinned in *Rnf20*^{fl/-}::*Nkx2.5*Cre⁺ embryos irrespective of comparable diameters to wildtype, and they exhibit large ventricular septal defects (left ventricle compact myocardium thickness p-value = 0.009, right ventricle compact myocardium thickness p-value = 0.004, septum length p-value = 0.0007) (**Fig. 1c**). We verified the *Nkx2.5*-specific deletion of *Rnf20* at e11.5; the RNF20 protein is in both myocardial and epicardial cells in the *Nkx2.5*Cre⁻ embryos, but only in the NKX2.5⁺ epicardial cells in *Nkx2.5*Cre⁺ embryos (**Fig. 1b**). While the majority of NKX2.5-expressing cells become cardiomyocytes, it remains possible that the structural defect phenotype results from a role for RNF20 in NKX2.5 expressing cardiac tissue including a subset of endocardial cells. Together, these data indicate that RNF20 is required in heart development in the FHF (NKX2.5-expressing cells), but is dispensable in the SHF (ISL1-expressing cells).

H2Bub1 is dynamically distributed during cardiomyocyte differentiation

Based on our data showing abnormal development of compact myocardium in response to tissue-specific *Rnf20* deletion, we investigated H2Bub1 during cardiomyocyte development using *in-vitro* differentiation of human induced pluripotent stem cells (iPSCs) into cardiomyocytes (CMs) (**online methods**)³⁸. To evaluate the genome-wide H2Bub1 profile and

subsequent transcriptional changes during CM development, ChIP-seq of H2Bub1 and bulk RNA-seq were performed simultaneously at five stages of CM differentiation: iPSCs, mesoderm (M), cardiac mesoderm (CMes), cardiac progenitor (CP) and CM. The stages of differentiation were verified through RNA-seq (**Fig. S3a, Supplemental Data 2**). H2Bub1 ChIP-seq peaks from iPSCs were grouped into four clusters based on H2Bub1 occupancy (high, moderate, low, and none) (**Fig. 2b, Supplemental Data 1**). ChIP-seq peaks from the other stages are displayed based on these clusters. The general profile of H2Bub1 is as previously reported, with very low-occupancy at the transcription start site (TSS) and coverage over the entire gene-body with gradual diminution from 5' to 3'¹⁷. The moderate-occupancy cluster has a distinct profile compared to the high and low-occupancy clusters. While the high and low-occupancy clusters have constant H2Bub1 throughout the 5' region of the gene-body, the moderate-occupancy cluster has a drop-off in H2Bub1 occupancy (**Fig. 2b**). Further, H2Bub1 occupancy increases between iPSC and M near the TSS, but remains constant between M and CMes. It then decreases between CMes and CP, and again remains constant between CP and CM (**Fig. 2a**).

We next performed DAVID gene ontology (GO) enrichment analysis on the genes in each cluster for each cell type to obtain their biological context. The high and moderate-occupancy clusters (Clusters 1 and 3) are enriched for general cell maintenance terms, showing a general need for H2Bub1 in maintaining homeostasis. The no-occupancy cluster (Cluster 4) is enriched for both general cell maintenance and chromatin assembly terms. Most interesting to us is the low-occupancy cluster (Cluster 2), which is enriched in development GO terms. Cluster 2 in iPSCs contains multiple types of developmental genes (muscle, renal, eye, heart, etc.), consistent with previous studies demonstrating RNF20 is required to exit pluripotency^{21,22}. Strikingly, as cells commit to a cardiac fate (in CP and CM), H2Bub1 is retained only on

developmental genes related to heart, nervous system, or appendage development (**Fig. S3b, S3c, Supplemental Data 1**).

Total H2Bub1 increases from CMes to CP, in contrast to the ChIP-seq data, which shows a decrease in H2Bub1 around gene bodies during the equivalent stages of CM differentiation. Total H2Bub1 in the other stages agree with the ChIP-seq data (**Fig. 2a, 2d, S3d**). One explanation is that H2Bub1 is decreasing in occupancy around the gene-body, but increasing in occupancy in heterochromatic regions. To test this, we compared the amount of H2Bub1 in the B compartment (heterochromatic compartment) to expected values calculated by generating bootstrap replicates from previously published data at each cell stage (**online methods**)³⁹. We found H2Bub1 first significantly decreases in the B compartment between iPSC and M, before significantly increasing as the cells progress through all the stages from M to CM (p-values $<1 \times 10^{-5}$). By the CM stage, there is significantly more H2Bub1 in the B compartment than expected by chance (p-values $<1 \times 10^{-5}$) (**Fig. S3e**). The gradual transition of H2Bub1 from gene bodies to heterochromatin is further supported by a significantly higher overlap of H2Bub1 and previously published H3K27me3 peaks (heterochromatic mark) on the same gene in CMs compared to iPSCs (p-values $<1 \times 10^{-5}$), versus no change in overlap of H2Bub1 and previously published H3K4me3 peaks (active genes)^{40,41}. Further, in CMs, there is a significantly higher overlap of H2Bub1 and H3K27me3 peaks on the same gene than of H2Bub1 and H3K4me3 peaks (p-value = 0.04). In iPSCs, both overlaps are equivalent (**Fig. S3f**). We conclude that the percent of H2Bub1 marks in active regions remains constant between iPSCs and CMs, but the percent of heterochromatic regions marked by H2Bub1 increases between iPSCs and CMs so that there are more heterochromatic regions marked by H2Bub1 than active regions at the CM stage. Together these data indicate that H2Bub1 increases in heterochromatic regions, which

accounts for the discrepancy between the observed increase in total H2Bub1 from CMes to CP, while gene-specific H2Bub1 decreases at the same stages.

H2Bub1 is selectively maintained on sarcomeric calcium genes

The distribution of many epigenetic marks is dynamic during development^{9,42}. To determine the temporal dynamics of H2Bub1 occupancy during cardiomyocyte differentiation, differential binding analysis on the H2Bub1 ChIP-seq data was performed during the progression from iPSCs to CMs. We found 316 regions that have increased H2Bub1 between iPSC and M, compared to 18 regions with decreased H2Bub1. Between M and CMes, there are 2 regions with decreased H2Bub1. The largest change in H2Bub1 happens upon transition from CMes to CP, with a decrease in 25,748 regions, while no gene-specific changes in H2Bub1 between CP and CM were observed (**Fig. 2f, Supplemental Data 1**). This drop off in H2Bub1 occupancy between CMes and CP is further shown in a Venn diagram comparing the unique CMes Ensembl genes near H2Bub1 regions to the unique CP genes (**Fig. 2e**). This is consistent with the overview data (**Fig. 2a**). DAVID GO enrichment analyses on regions that change in H2Bub1 occupancy, based on the average of all three replicates, indicate that they are mostly cell maintenance genes (**Fig. 2g**). Since this mark is thought to be activating, we verified expression of the genes near H2Bub1 for all cell stages. We evaluated the overlap of ChIP-seq and RNA-seq results in the terminal CM stage in more detail and identified 432 genes that are predicted to be direct targets of H2Bub1 (**Fig. 2c**). Consistent with a role for H2Bub1 in CMs, DAVID GO analysis of these 432 genes identified a significant enrichment (p-value = 0.014) in cardiac conduction genes, including the calcium channel *CACNA1C*.

Given this connection between H2Bub1 and cardiac function genes, we hypothesized that the genes that maintain their mark during the large drop off in H2Bub1 occupancy between CMes and CP are also cardiac genes. Genes that maintain H2Bub1 in CP are enriched for calcium signaling genes (**Fig. 2h**). Interestingly, calcium signaling genes remain constant in H2Bub1 occupancy over time, unlike housekeeping genes which follow the general trend outlined in Fig. 2a (**Fig. 2i**). Further, a significantly higher proportion of calcium signaling genes is identified within the genes that have maintained H2Bub1 compared to genes with downregulated H2Bub1 (Z score = 4.3996, p-value = 1.1×10^{-5}). Importantly, ten of the eleven calcium signaling genes that maintain H2Bub1 are associated with cardiomyopathy either through patient mutations or mouse models (**Fig. 2h**)⁴³⁻⁵². These include *CACNA1C* and *RYR2* which have been linked to left ventricular non-compaction (LVNC)^{51,52}. Together, these data indicate that H2Bub1 is selectively maintained on tissue-specific genes in CMs to promote their expression.

Patterns in H2Bub1 occupancy predict gene expression

The distribution of chromatin marks, such as H3K4me3, is known to affect gene expression, so we wanted to determine if the same is true for H2Bub1²⁸. We next asked if we could identify patterns of H2Bub1 distribution and amount on CMes marked genes that we could use to predict the subsequent loss of H2Bub1 and gene expression in CP. By using a previously established method, we identified multiple groups of genes (patterns) that have similar distribution or amount of H2Bub1 across the gene body (**Fig. S4a, Supplemental Data 1**)⁵³. Interestingly, the calcium signaling genes were among the many genes that did not get placed into any pattern, hereafter referred to as unpatterned genes.

We next calculated the ratio of H2Bub1 peaks that decrease in occupancy between CMes and CP to total H2Bub1 peaks for each of these patterns. Patterned genes have a higher ratio of peaks decreasing in occupancy than average (**Fig. S4b**). On the contrary, some unpatterned genes, such as the calcium signaling genes, maintain their H2Bub1 occupancy. The decreased occupancy in patterned genes corresponds to their significantly decreased expression in CP compared to CMes (all p-values < 0.05). Thus, if H2Bub1 only occupies a particular region of a gene body (such as in housekeeping genes), it will likely decrease in H2Bub1 occupancy and gene expression between CMes and CP. In contrast, if H2Bub1 is relatively even throughout the gene body (such as in calcium signaling genes), it will instead maintain H2Bub1 occupancy and gene expression (**Fig. S4c, 2i**). This implies that patterned H2Bub1 marks precede loss of the mark and decreased expression, while evenly distributed H2Bub1 marks are retained and predict continued expression.

Mutations in the RNF20-complex affect cardiomyocyte differentiation

Since H2Bub1 is selectively maintained on sarcomeric calcium signaling genes linked to cardiomyopathy, along with the known link between ubiquitinase complex members (*RNF20* and *UBE2B*) and CHD, we next asked how defective H2Bub1 impacts CM development. To address this, we first established iPSC lines with mutations affecting *RNF20*, and evaluated how the mutations impact the capacity for iPSCs to differentiate into CMs. In each of two independent CRISPR experiments, we created one *RNF20*^{+/-} iPSC line. The two independent iPSC lines have different mutations, and are both predicted to be functionally haploinsufficient trans-heterozygotes. For simplicity, we will be referring to these iPSC lines collectively as *RNF20*^{+/-}

(**Fig. S5a, online methods**). *RNF20* knockdown was verified by western blotting for RNF20 (**Fig. 4i, S6d**).

RNF20^{+/-} and wild-type iPSCs were then simultaneously differentiated into CMs³⁸. At iPSC, M, and CM stages (when the cells are relatively homogenous), we performed immunofluorescence for markers of pluripotency (OCT4), M (Brachyury), CMes (NKX2.5), CP (ISL1), and CM (cardiac troponin T, TNNT)³⁸. At the iPSC stage, both wild-type and *RNF20*^{+/-} cells express OCT4, indicating that haploinsufficiency for *RNF20* does not affect pluripotency (**Fig. S7a**). At the M stage, again both wild-type and *RNF20*^{+/-} cells express Brachyury, but only wild-type cells lose their OCT4 expression, suggesting the *RNF20*^{+/-} cells have a delay in exiting pluripotency (**Fig. S7b**).

Wild-type CMs beat, no longer express Brachyury, and instead express NKX2.5, ISL1, and TNNT. However, most cells in both *RNF20*^{+/-} lines continue to express Brachyury, and have no NKX2.5 or TNNT expression. Interestingly, the *RNF20*^{+/-} cells do express ISL1, a pan-cardiac marker, despite not expressing NKX2.5 (**Fig. S7c**). Since ISL1 is expressed in all CP cells, this may indicate that *RNF20*^{+/-} cells are in a less mature state^{54,55}. Importantly, unlike wild-type cells, most *RNF20*^{+/-} cells fail to beat (even by day 20) (**Fig. 3a, movie 1, movie 2, movie 3, movie 4**). In conclusion, the *RNF20*^{+/-} cell lines fail to differentiate into normal CMes, and thus ultimately fail to make beating CMs.

To further investigate the possible role for the RNF20-complex on CM differentiation, we also established iPSC lines with mutations affecting *UBE2B*, and evaluated how the mutations impact the capacity for iPSCs to differentiate into CMs. In each of two independent CRISPR experiments, we created one *UBE2B*^{-/-} iPSC lines. Since UBE2A and UBE2B are more than 95% identical at the protein level, these knockouts were validated by sequencing the cDNA

(**Fig. S8a, online methods**)⁵⁶. Mutant and wild-type iPSCs were then simultaneously differentiated into CMs³⁸. As with the *RNF20*^{+/-} iPSCs, *UBE2B*^{-/-} iPSCs mirror wild-type, but by M stage, most *UBE2B*^{-/-} cells gain Brachyury expression, while retaining OCT4 expression (**Fig. S7a, S7b**).

In contrast to the *RNF20*^{+/-} cell lines, about a third of *UBE2B*^{-/-} cells (*UBE2B*^{-/-}1 25/72, *UBE2B*^{-/-}2 24/69) beat at the CM stage (**Fig. 3a, movie 1, movie 5, movie 6, movie 7, movie 8**). Given this heterogeneity, we evaluated both beating (lactate selection) and non-beating (no lactate selection) cells at a time corresponding to the wild-type CM stage (**Online Methods**). The beating *UBE2B*^{-/-} cells are mostly Brachyury negative, and NKX2.5, ISL1, and TNNT positive, while the non-beating *UBE2B*^{-/-} cells are mostly Brachyury and ISL1 positive, and NKX2.5 and TNNT negative (**Fig. S7c**). These data indicate that *UBE2B*^{-/-} iPSCs can differentiate into beating CMs, but do so at a reduced efficiency compared to wild-type iPSCs. Additionally, non-beating *UBE2B*^{-/-} cells are phenotypically equivalent to *RNF20*^{+/-} cells. Collectively, these data demonstrate a requirement for the RNF20-complex in normal CM differentiation.

RNF20-complex component dosage determines level of H2Bub1 deposition

To relate the dynamic nature of H2Bub1 over CM differentiation and mouse heart development to the ubiquitination complex (RNF20-RNF40-UBE2B and the scaffolding protein WAC), we examined the expression of the complex components. To do this, we first asked how the expression of the RNF20-complex changes over heart development *in-vivo*. Protein levels of RNF20-complex members are dynamic between embryonic day 9.5 (e9.5) and adult mouse hearts. RNF20 peaks at e11.5 and precipitously drops after birth, while RNF40 peaks at e14.5

and also precipitously drops after birth. UBE2A/B increases up until adulthood. Importantly, changes in these complex components affect H2Bub1 deposition; H2Bub1 is lowest at e9.5 and highest in adult mice (**Fig. S1e**). These observations suggest that the ratio of the RNF20-complex components may play an important role in H2Bub1 deposition efficiency.

We then determined the trajectory of RNF20-complex mRNA during CM differentiation using RNA-seq data. *UBE2B* and *RNF40* are relatively constant over CM differentiation, while *WAC* reaches a maximum and *RNF20* reaches a minimum at the CP stage (**Fig. 4a**). Protein levels do not directly correlate with mRNA; RNF20, RNF40, and UBE2A/B proteins all peak at the CP stage, before falling to very low levels at the CM stage (**Fig. 4b, S6a**). The differences in mRNA and protein levels could be secondary to persistence of long-lived protein from earlier developmental time points, or from post-transcriptional regulation. In addition, UBE2B levels reflect both UBE2B and UBE2A, which are >95% identical at the protein level, and cannot be discriminated by Western blotting⁵⁶. The dynamic levels of RNF20-complex components and lack of concordance of any individual complex component to the resulting H2Bub1 levels suggest that deposition efficiency may depend on the ratio of the components, as it does in yeast. Previous work demonstrated that a higher amount of Lge1 (corresponding to WAC) relative to Bre1 (corresponding to RNF20/RNF40) leads to a better complex condensate, and is predicted to more efficiently deposit H2Bub1¹⁶. Therefore, we would predict that at the CP stage, when *WAC* is most highly expressed, H2Bub1 would be the highest, consistent with our observation (**Fig. 2d, 4c, S3d**). Our data both confirm and expand these findings to mammalian cells. In conclusion, as the ratios of the complex components change, the total H2Bub1 changes in response (**Fig. 4d**).

To test this further, we asked how global H2Bub1 deposition changes in our iPSC and mouse RNF20-complex mutants. Analysis of the *RNF20*^{+/-} cell lines was limited to the iPSC and M stages, since they fail to differentiate further. As expected *RNF20*^{+/-} iPSCs have decreased *RNF20* mRNA and protein, but also have upregulated *RNF40* mRNA and protein, and decreased *WAC*, perhaps to attempt to correct the abnormal ratio (**Fig. 4h, 4i, S6d, S6f**). Interestingly, these cells also have a paradoxical increase in total H2Bub1 (**Fig. 4j, S6e**). We propose that the abnormal ratio of complex components may increase the efficiency of the complex, leading to the paradoxical increase in the amount of H2Bub1 (**Fig. 4k**). We evaluated the genome-wide H2Bub1 binding profile and gene expression in *RNF20*^{+/-} iPSCs (**S5b, Supplemental Data 3, Supplemental Data 4**). Consistent with the increased total H2Bub1 levels, H2Bub1 near gene bodies is also increased and correlates with transcriptional changes (**Fig. 4j, 5a, 5b, 5c, S6e**). DAVID GO enrichment analysis on genes with increased H2Bub1 occupancy and mRNA expression indicate that many of these genes are involved with transcription, splicing, and/or DNA and protein modifications (**Fig. 5d**). Since these classes of genes broadly affect downstream transcription, haploinsufficiency for *RNF20* likely leads to pleiotropic effects.

By M stage, *RNF20* mRNA is restored to normal levels in *RNF20*^{+/-} cells, but RNF20 protein remains decreased compared to wild-type (**Fig. 4h, 4i, S6d, S6f**). This suggests attempted autoregulation of RNF20, since RNF20 itself has increased H2Bub1 in both *RNF20*^{+/-} iPSC lines compared to wild-type iPSCs (**Supplemental Data 3**). As in the *RNF20*^{+/-} iPSCs, *WAC* mRNA is also decreased (**Fig. 4h, S6f**). The genome-wide H2Bub1 binding profile and gene expression in *RNF20*^{+/-} M cells indicate that *RNF20* haploinsufficiency causes pleiotropic responses upon exiting pluripotency (**Supplemental Data 3, Supplemental Data 4**). There is a lack of genes with significant differential H2Bub1 occupancy in *RNF20*^{+/-} cells compared to wild-type, even

though there is an overall decrease in global H2Bub1 (**Fig. S5c, S5d, Supplemental Data 3**). By the time the *RNF20*^{+/-} cells reach the M stage, their H2Bub1 levels deviate so far from normal levels that there is catastrophic dysregulation of gene expression, which prevents the cells from continuing to differentiate.

UBE2B^{-/-} cell lines have reduced RNF20 and RNF40 levels at both iPSC and M stages, possibly due to decreased H2Bub1 on these genes, resulting in transcriptional downregulation (**Fig. 4e, S6b**). As expected, loss of UBE2B results in decreased total H2Bub1 at the iPSC and M stages (**Fig. 4f, S6c**). The small amount of residual H2Bub1 is likely retained in *UBE2B*^{-/-} cell lines due to the presence of UBE2A, which has been shown to be partially redundant with UBE2B with regard to H2Bub1^{14,56}. Partial compensation of UBE2A by UBE2B is also supported by the observation that *Ube2b*^{-/-} and *Ube2a*^{-/-} mice have abnormal fertility, but can survive to adulthood^{57,58}, while *Rnf20*^{-/-} mice are pre-implantation lethal.

Next we asked how H2Bub1 changes in e12.25 embryonic mouse hearts in response to FHF-specific deletion of Rnf20 (*Rnf20*^{fl/-}::*Nkx2.5Cre*⁺). Both RNF20 and H2Bub1 are reduced in *Rnf20*^{fl/-}::*Nkx2.5Cre*⁺ mouse embryo hearts. The residual H2Bub1 and RNF20 is perhaps due to the heterogeneity of the whole heart tissue, which includes both NKX2.5⁺ (*Rnf20*-null) and NKX2.5⁻ (*Rnf20*-wild-type) cells (**Fig. 4l, 4m, S6g**).

Together, our data suggest that partial and complete loss of *RNF20* differentially affect H2Bub1. RNF20 haploinsufficiency raises the RNF20-complex efficiency, which results in increased H2Bub1 (**Fig. 4k**). Complete loss of either RNF20 or UBE2B leads to a failure to form a functional RNF20-complex, and reduction in total H2Bub1 (**Fig. 4g, 4n**). Thus, we propose that dynamic changes in ratios of the RNF20-complex components over developmental time

create a mechanism to precisely modulate H2Bub1 patterns and temporally regulate H2Bub1-dependent gene transcription (**Fig. 4d**).

Total H2Bub1 reduction decreases sarcomeric calcium signaling gene expression *in-vitro* and *in-vivo*

In contrast to *RNF20*^{+/-} cells, the *UBE2B*^{-/-} cells have decreased total H2Bub1 and are able to form beating CMs. To understand how reduced H2Bub1 could alter CM gene expression, we performed RNA-seq analysis of beating *UBE2B*^{-/-} CMs, and compared them to time-matched wild-type iPSC-derived CMs (**Fig. 6a, S8b**). Differential expression analysis identified 1393 downregulated and 1555 upregulated transcripts that are shared between both independent *UBE2B*^{-/-} cell lines compared to wild-type (**Supplemental Data 6**). To obtain a biological context for the genes with decreased expression, we did DAVID GO enrichment analysis. About half of the significant GO terms (37/76) are related to sarcomeric calcium genes, sarcomere genes, and/or cardiomyopathy genes. Most notably, two genes with decreased expression, *CACNA1C* and *RYR2*, are also amongst the genes with selectively maintained H2Bub1 upon wild-type transition from CMes to CP (**Fig. 6c**).

To determine if this *in-vitro* mechanism also functions *in-vivo*, we analyzed expression of the embryonic sarcomeric calcium signaling genes (*Cacna1c*, *Ryr2*, *Ncx*, and *Serca2a*) at e11.5 in *Rnf20*^{fl/+}::*Nkx2.5Cre*⁺ and *Rnf20*^{fl/-}::*Nkx2.5Cre*⁺ embryonic hearts, prior to any visible cardiac defects (**Fig. 6a**). We observed that the *Rnf20*^{fl/-}::*Nkx2.5Cre*⁺ have significantly lower *Cacna1c* and *Serca2a* expression than *Rnf20*^{fl/+}::*Nkx2.5Cre*⁺ siblings (*Cacna1c* p-value = 0.03, *Serca2a* p-value = 0.001) (**Fig. 6b**). Thus, RNF20-complex dependent H2Bub1 is necessary for normal sarcomeric calcium gene expression in iPSC-derived CMs and in mice.

Sarcomeres are abnormal when total H2Bub1 is reduced

Our results showing a decrease in cardiac sarcomeric gene expression in *UBE2B*^{-/-} CMs, along with their beating inefficiency, led us to evaluate the effect of reduced H2Bub1 on cardiac sarcomere structure *in-vivo*. Transmission electron microscopy revealed a missing H zone in e12.25 *Rnf20*^{flx/-}::*Nkx2.5*Cre⁺ mouse sarcomeres (**Fig. 3b**). The H zone is the region of the sarcomere that is devoid of actin filaments. At the center of the H zone is the M band, consisting mostly of myomesin at e12.25, which functions to anchor the filaments to titin. Due to an elastic domain in the middle of the embryonic splice variant of myomesin, this structure is not able to be seen on TEM at this stage. An abnormal M band will lead to abnormal sarcomere organization, as observed in the *Rnf20*^{flx/-}::*Nkx2.5*Cre⁺ mice⁵⁹. Interestingly, our RNA-seq data show that two components of the M-band (Myomesin and Titin) and two proteins required for regulating their alternative splicing (*RBM20* and *RBM24*) have decreased expression in the *UBE2B*^{-/-} CMs compared to the wild-type (median b value = -1.54)^{60,61}. This indicates that H2Bub1 regulates M band proteins, which are required for normal sarcomeric structure and beating efficiency.

Accumulation of H2Bub1 near the center of tissue-specific genes correlates with enhanced efficiency of transcriptional elongation

Strikingly, while loss of *UBE2B* leads to decreased total H2Bub1 levels, ChIP-seq of CMs comparing H2Bub1 between wild-type and *UBE2B*^{-/-} cells demonstrates that gene-specific H2Bub1 is only decreased in 8 Ensembl genes (**Fig. 4f, S6c, S8c, Supplemental Data 5**). To identify differences in H2Bub1 patterns between *UBE2B*^{-/-} and wild-type CMs, we identified calcium and/or sarcomeric genes with reduced mRNA in *UBE2B*^{-/-} CMs, along with calcium signaling genes that selectively maintain H2Bub1 between CMs and CP. We then created

metagenes by combining our selected genes ChIP-seq data and averaging the replicates, allowing us to visualize common features of H2Bub1 for those gene sets (calcium genes $n = 28$, sarcomere genes $n = 70$, **Supplemental Data 5**). Wild-type cells have an accumulation of H2Bub1 near the center of the metagenes, but this accumulation is either reduced or completely absent in *UBE2B*^{-/-} cells (**Fig. 7a, 7b**). To validate that this accumulation is not a technical artifact, we generated 30 sets of “random” quantity and sized-matched gene sets for both the calcium and sarcomeric genes. To control for the expression differences in these genes, we chose an even distribution of genes sets that were upregulated, downregulated, or unchanged between both mutants compared to wild-type. While accumulation is present near the center of some of these metagenes, none of them show differential H2Bub1 occupancy at the accumulation region between mutant and wild-type cells. We provide one representative graph (**Fig. 7c**). We next determined whether this phenomenon is true for all genes, or just tissue-specific long genes by producing metagene plots for all short genes (less than 100 bp) and all long genes (greater than 10 Kb). We conclude that H2Bub1 accumulation near the center of genes is present in all long genes, but UBE2B-dependent accumulation is unique to tissue-specific long genes (**Fig. S8d, S8e**). Therefore, the H2Bub1 accumulation near the center of the gene is specific only to certain gene classes, including calcium and sarcomeric genes.

Since the RNF20-complex is known to be involved in transcriptional elongation and H2Bub1 is found on long tissue-specific genes during left-right patterning of the heart, we hypothesized that the accumulation of H2Bub1 near the center of long genes may support their transcriptional elongation (median length of all genes is 31 Kb, compared to 456 and 69 Kb for calcium signaling and sarcomeric genes, respectively). To test this hypothesis, we asked whether there was loss of the 3' end of transcripts in *UBE2B*^{-/-} CMs compared to wild-type. When

comparing the ratio of mutant to wild-type RNA in both the calcium and sarcomeric gene sets, transcripts are less abundant in *UBE2B*^{-/-} cells in the most 3' 20% of the gene indicating inefficient transcription elongation in the mutants; example transcript traces for *CACNA1C* and *RYR2* are shown (**Fig. 7d, 7e, 7g**). To validate this conclusion, we repeated the same analysis on our “random” gene sets which only had transcriptional efficiency drop-off in the last 5-10% of the 3' end. We provide one representative graph (**Fig. 7f**). Thus, these data support the conclusion that accumulation of H2Bub1 near the center of calcium and sarcomeric genes is UBE2B dependent and correlates with enhanced transcription elongation efficiency.

We next asked if this H2Bub1 accumulation is unique to CMs. To address this, we used previously published H2Bub1-ChIP-seq data of wild-type mESCs and MEFs³³. We determined the MEF-specific genes by identifying differentially expressed genes between MEFs and mESCs; they are enriched in adhesion (n = 143), extra-cellular matrix (ECM) (n = 130), and ions (n = 160) GO terms (**Supplemental Data 5**). Similar to the CM tissue-specific genes, these genes are also long (median length is 43 Kb for adhesion, 48 Kb for ECM, and 50 Kb for ions). The metagene plots generated for adhesion and ECM show an accumulation of H2Bub1 near the center of the gene, similar to the calcium and sarcomeric genes in CMs. Additionally, this accumulation was higher in the tissue with increased expression of the differentially expressed genes (MEFs), as expected (**Fig. 7h, 7i**). As in CMs, all of the quantity and size-matched “random” genes sets generated show no accumulation of H2Bub1 near the center of the gene, as shown in one representative graph (**Fig. 7j**). The CM and MEF data collectively suggest that accumulation of H2Bub1 near the center of tissue-specific long genes is a general mechanism for regulation of transcriptional efficiency. In CMs, central H2Bub1 accumulation and efficient

transcription of sarcomeric and calcium signaling genes depend on UBE2B, providing a mechanism to explain the cardiac phenotype in the *Rnf20^{fl/fl}::Nkx2.5Cre* mouse model

Discussion

Together, our data support a requirement for tight control of H2Bub1 levels in cardiac development. Increased total H2Bub1 leads to failure to form CMs, while decreased total H2Bub1 reduces the efficiency of CM differentiation *in-vitro*, and leads to reduced expression of calcium signaling genes and structural abnormalities of the cardiac sarcomere, including a deficient H zone, *in-vivo* (**Fig. 8a**). H2Bub1 is highly dynamic during CM differentiation: early, the mark is increased and then decreased on housekeeping genes, and later the mark is selectively maintained on calcium signaling genes, while it shifts from euchromatic to heterochromatic regions (**Fig. 8b**). Finally, we show that the shape of the H2Bub1 mark changes between wild-type and UBE2B^{-/-} CMs. The profile of H2Bub1 on short genes is as previously reported, with coverage over the entire gene-od higher at the 5' end than the 3' end of the gene¹⁷. Notably, long genes have a vastly different profile: there is tissue-specific UBE2B-dependent H2Bub1 accumulation near the center of the gene, which correlates with efficient transcriptional elongation (**Fig. 8c**).

H2Bub1 is an enigmatic chromatin mark localizing to the entire gene body, instead of discrete promoter and enhancer regions. At the onset of CM differentiation, we highlight an increase in both the total and gene-specific H2Bub1, as seen previously in neuronal cells and keratinocytes²¹. Further, our data support a model where tissue-specific transcriptional effects are determined by the retention, instead of deposition, of H2Bub1 on tissue-specific genes. H2Bub1 has been linked to transcriptional elongation, but how it affects transcriptional elongation is

unclear^{13,14,23,30,32,33,62}. We observed accumulation of H2Bub1 near the center of a subset of long genes. Interestingly, it was previously determined that RNA Pol II exhibits a similar accumulation at the center of all expressed genes⁶³. Since H2Bub1 co-localizes with RNA Pol II, the published RNA Pol II distribution is in concordance with the H2Bub1 distribution³⁰. We propose that H2Bub1 enhances transcriptional elongation of long tissue-specific genes. This conclusion is supported by previous work finding that long genes are more likely to depend on RNF20 to be induced upon differentiation into neuronal cells²¹. Further evidence is the presence of H2Bub1 on cilia genes (which have an average length of 73 Kb) in the multi-ciliated cells of oviducts (which require expression of long motile cilia genes), but not in the liver cells, which do not¹⁸. Combined with our current data analyzing CMs and MEFs, we propose that targeted H2Bub1 accumulation is a more general mechanism regulating tissue-specific transcriptional elongation efficiency on long genes.

Ratios of RNF20-complex components affect the efficiency of H2Bub1 deposition. We show that if *Rnf20* is deleted, total H2Bub1 decreases; if *RNF20* is haploinsufficient, total H2Bub1 paradoxically increases; if *UBE2B* is deleted, total H2Bub1 decreases. The varying levels of H2Bub1 resulting from altered levels of distinct RNF20-complex components support an existing hypothesis, whereby the exact stoichiometric ratios of the complex components affect its efficiency¹⁶. Thus, missing a component will yield the expected results of no H2Bub1, but having less of a component will affect the ratios of the complex components, altering the efficiency. We suggest that iPSCs normally maintain complex components in a less efficient ratio to constrain the level of H2Bub1 to maintain pluripotency.

We propose that heart development is exquisitely sensitive to H2Bub1 dosage. The difference in phenotype between the mice with RNF20 deleted in the FHF and SHF is striking,

suggesting that the SHF-derived tissues (mostly endocardium and epicardium) are less sensitive to altered H2Bub1 dosage than the FHF-derived tissues (mostly cardiomyocytes)^{64,65}. Since RNF20 is required to exit pluripotency, the presence of H2Bub1 on a subset of genes is crucial for this process to occur. Once the cells exit pluripotency, these marks become restricted to heart-specific genes. If there is an abnormal amount of H2Bub1 upon exiting pluripotency, the cells are unable to fully differentiate. Our data indicate that RNF20-complex-dependent H2Bub1 is necessary for normal cardiac development through regulating the transcriptional elongation efficiency of cardiac calcium signaling and sarcomeric genes during CM differentiation and development.

The question remains how H2Bub1 affects development of cardiac structure, since both human patients with mutations affecting H2Bub1 and mouse embryos with cardiac-specific deletion of *Rnf20* have structural heart defects. A possible link to the observed structural heart defects in mice is that altered expression of calcium signaling genes and abnormal sarcomeric structure observed in *Rnf20*^{fl/-}::*Nkx2.5Cre*⁺ mouse embryos led to defective cardiac function during embryonic development, and that the resulting hemodynamic derangement affects structural cardiac morphogenesis. Extensive evidence supports interdependence between embryonic hemodynamics and valve development, cardiac trabeculation, myocardial proliferation and formation of the epicardium (reviewed in ⁶⁶). Genomic studies of human CHD patients are beginning to provide further evidence of an overlap between genes classically linked to cardiomyopathy, and patients presenting with structural CHD. For example, dominant mutations affecting myosin heavy chain 6 (*MYH6*) are associated with cardiomyopathy and atrial septal defects^{67,68}, while recessive mutations in *MYH6* are found in 11% of patients with Shone syndrome, characterized by valve defects and multiple levels of left ventricular obstruction³. The

essential role of H2Bub1 in CM development and cardiac morphogenesis in mouse and human provide further support for genetic overlap between cardiac structure development and myocardial function, and suggest that a subset of patients with structural heart defects caused by genetic defects affecting cardiomyocytes may be more vulnerable to myocardial dysfunction. Although there are likely variations in the absolute H2Bub1 levels required for normal iPSC-derived CM development, mouse embryo, and human heart development, our observations in iPSC-derived CMs and mouse embryos indicate a shared requirement for precise control of H2Bub1 in the heart.

Acknowledgements

We thank the Center for Cellular and Molecular Imaging at Yale for transmission electron microscopy and the Developmental Histology Tissue Services at Yale for adult mouse heart histology. We would like to thank Dr. Jon Seidman and Dr. Christine Seidman for their assistance with the human iPSC work. We would also like to thank Dr. Yibing Qyang and Dr. William Pu for sharing mouse lines with us. This work was supported by NSFGRFP to SB, and R35HL145249 and 2U01HL098162 to MB.

Author Contributions

SB and MB conceived and designed the experiments. SB, JD, KB, IBB, LK, and MB performed the experiments in mouse. SB and LW performed the experiments in the iPSCs. SB performed the bioinformatics analysis of the ChIP-seq and RNA-seq experiments, and SC provided conceptual advice on the analysis. SB and MB wrote the manuscript, which was read and approved by all of the authors.

555 References

- 556 1. Pierpont, M.E. *et al.* Genetic Basis for Congenital Heart Disease: Revisited: A Scientific
557 Statement From the American Heart Association. *Circulation* **138**, e653-e711 (2018).
- 558 2. Homsy, J. *et al.* De novo mutations in congenital heart disease with neurodevelopmental
559 and other congenital anomalies. *Science* **350**, 1262-6 (2015).
- 560 3. Jin, S.C. *et al.* Contribution of rare inherited and de novo variants in 2,871 congenital
561 heart disease probands. *Nat Genet* **49**, 1593-1601 (2017).
- 562 4. Zaidi, S. *et al.* De novo mutations in histone-modifying genes in congenital heart disease.
563 *Nature* **498**, 220-3 (2013).
- 564 5. Ang, S.Y. *et al.* KMT2D regulates specific programs in heart development via histone H3
565 lysine 4 di-methylation. *Development* **143**, 810-21 (2016).
- 566 6. Jones, B. *et al.* The histone H3K79 methyltransferase Dot1L is essential for mammalian
567 development and heterochromatin structure. *PLoS Genet* **4**, e1000190 (2008).
- 568 7. Montgomery, R.L. *et al.* Histone deacetylases 1 and 2 redundantly regulate cardiac
569 morphogenesis, growth, and contractility. *Genes Dev* **21**, 1790-802 (2007).
- 570 8. Van Laarhoven, P.M. *et al.* Kabuki syndrome genes KMT2D and KDM6A: functional
571 analyses demonstrate critical roles in craniofacial, heart and brain development. *Hum*
572 *Mol Genet* **24**, 4443-53 (2015).
- 573 9. Wamstad, J.A. *et al.* Dynamic and coordinated epigenetic regulation of developmental
574 transitions in the cardiac lineage. *Cell* **151**, 206-20 (2012).
- 575 10. Robzyk, K., Recht, J. & Osley, M.A. Rad6-dependent ubiquitination of histone H2B in
576 yeast. *Science* **287**, 501-4 (2000).
- 577 11. Wood, A. *et al.* Bre1, an E3 ubiquitin ligase required for recruitment and substrate
578 selection of Rad6 at a promoter. *Mol Cell* **11**, 267-74 (2003).
- 579 12. Kim, J., An, Y.K., Park, S. & Lee, J.S. Bre1 mediates the ubiquitination of histone H2B by
580 regulating Lge1 stability. *FEBS Lett* **592**, 1565-1574 (2018).
- 581 13. Kim, J., Hake, S.B. & Roeder, R.G. The human homolog of yeast BRE1 functions as a
582 transcriptional coactivator through direct activator interactions. *Mol Cell* **20**, 759-70
583 (2005).
- 584 14. Kim, J. *et al.* RAD6-Mediated transcription-coupled H2B ubiquitylation directly
585 stimulates H3K4 methylation in human cells. *Cell* **137**, 459-71 (2009).
- 586 15. Zhang, F. & Yu, X. WAC, a functional partner of RNF20/40, regulates histone H2B
587 ubiquitination and gene transcription. *Mol Cell* **41**, 384-97 (2011).
- 588 16. Gallego, L.D. *et al.* Phase separation directs ubiquitination of gene-body nucleosomes.
589 *Nature* **579**, 592-597 (2020).
- 590 17. Jung, I. *et al.* H2B monoubiquitylation is a 5'-enriched active transcription mark and
591 correlates with exon-intron structure in human cells. *Genome Res* **22**, 1026-35 (2012).
- 592 18. Robson, A. *et al.* Histone H2B monoubiquitination regulates heart development via
593 epigenetic control of cilia motility. *Proc Natl Acad Sci U S A* **116**, 14049-14054 (2019).
- 594 19. VanDusen, N.J. *et al.* Massively parallel in vivo CRISPR screening identifies RNF20/40 as
595 epigenetic regulators of cardiomyocyte maturation. *Nat Commun* **12**, 4442 (2021).
- 596 20. Chen, S., Li, J., Wang, D.L. & Sun, F.L. Histone H2B lysine 120 monoubiquitination is
597 required for embryonic stem cell differentiation. *Cell Res* **22**, 1402-5 (2012).

- 598 21. Fuchs, G. *et al.* RNF20 and USP44 regulate stem cell differentiation by modulating H2B
599 monoubiquitylation. *Mol Cell* **46**, 662-73 (2012).
- 600 22. Karpiuk, O. *et al.* The histone H2B monoubiquitination regulatory pathway is required
601 for differentiation of multipotent stem cells. *Mol Cell* **46**, 705-13 (2012).
- 602 23. Shema, E., Kim, J., Roeder, R.G. & Oren, M. RNF20 inhibits TFIIIS-facilitated
603 transcriptional elongation to suppress pro-oncogenic gene expression. *Mol Cell* **42**, 477-
604 88 (2011).
- 605 24. Shema, E. *et al.* The histone H2B-specific ubiquitin ligase RNF20/hBRE1 acts as a
606 putative tumor suppressor through selective regulation of gene expression. *Genes Dev*
607 **22**, 2664-76 (2008).
- 608 25. Tarcic, O. *et al.* RNF20 Links Histone H2B Ubiquitylation with Inflammation and
609 Inflammation-Associated Cancer. *Cell Rep* **14**, 1462-76 (2016).
- 610 26. Wang, E. *et al.* Histone H2B ubiquitin ligase RNF20 is required for MLL-rearranged
611 leukemia. *Proc Natl Acad Sci U S A* **110**, 3901-6 (2013).
- 612 27. Lee, J.S. *et al.* Histone crosstalk between H2B monoubiquitination and H3 methylation
613 mediated by COMPASS. *Cell* **131**, 1084-96 (2007).
- 614 28. Xie, W. *et al.* RNF40 regulates gene expression in an epigenetic context-dependent
615 manner. *Genome Biol* **18**, 32 (2017).
- 616 29. Henry, K.W. *et al.* Transcriptional activation via sequential histone H2B ubiquitylation
617 and deubiquitylation, mediated by SAGA-associated Ubp8. *Genes Dev* **17**, 2648-63
618 (2003).
- 619 30. Fuchs, G., Hollander, D., Voichek, Y., Ast, G. & Oren, M. Cotranscriptional histone H2B
620 monoubiquitylation is tightly coupled with RNA polymerase II elongation rate. *Genome*
621 *Res* **24**, 1572-83 (2014).
- 622 31. Kim, J. & Roeder, R.G. Direct Bre1-Paf1 complex interactions and RING finger-
623 independent Bre1-Rad6 interactions mediate histone H2B ubiquitylation in yeast. *J Biol*
624 *Chem* **284**, 20582-92 (2009).
- 625 32. Pavri, R. *et al.* Histone H2B monoubiquitination functions cooperatively with FACT to
626 regulate elongation by RNA polymerase II. *Cell* **125**, 703-17 (2006).
- 627 33. Wang, F. *et al.* Histone H2Bub1 deubiquitylation is essential for mouse development,
628 but does not regulate global RNA polymerase II transcription. *Cell Death Differ* (2021).
- 629 34. Valenzuela, D.M. *et al.* High-throughput engineering of the mouse genome coupled with
630 high-resolution expression analysis. *Nat Biotechnol* **21**, 652-9 (2003).
- 631 35. Xu, Z. *et al.* H2B ubiquitination regulates meiotic recombination by promoting chromatin
632 relaxation. *Nucleic Acids Res* **44**, 9681-9697 (2016).
- 633 36. Moses, K.A., DeMayo, F., Braun, R.M., Reecy, J.L. & Schwartz, R.J. Embryonic expression
634 of an Nkx2-5/Cre gene using ROSA26 reporter mice. *Genesis* **31**, 176-80 (2001).
- 635 37. Pfaff, S.L., Mendelsohn, M., Stewart, C.L., Edlund, T. & Jessell, T.M. Requirement for LIM
636 homeobox gene *Isl1* in motor neuron generation reveals a motor neuron-dependent
637 step in interneuron differentiation. *Cell* **84**, 309-20 (1996).
- 638 38. Lian, X. *et al.* Directed cardiomyocyte differentiation from human pluripotent stem cells
639 by modulating Wnt/beta-catenin signaling under fully defined conditions. *Nat Protoc* **8**,
640 162-75 (2013).

39. Bertero, A. *et al.* Dynamics of genome reorganization during human cardiogenesis reveal an RBM20-dependent splicing factory. *Nat Commun* **10**, 1538 (2019).
40. Inoue, F. *et al.* A systematic comparison reveals substantial differences in chromosomal versus episomal encoding of enhancer activity. *Genome Res* **27**, 38-52 (2017).
41. Kazachenka, A. *et al.* Identification, Characterization, and Heritability of Murine Metastable Epialleles: Implications for Non-genetic Inheritance. *Cell* **175**, 1259-1271 e13 (2018).
42. Mei, H. *et al.* H2AK119ub1 guides maternal inheritance and zygotic deposition of H3K27me3 in mouse embryos. *Nat Genet* **53**, 539-550 (2021).
43. Tang, Y., Tian, X., Wang, R., Fill, M. & Chen, S.R. Abnormal termination of Ca²⁺ release is a common defect of RyR2 mutations associated with cardiomyopathies. *Circ Res* **110**, 968-77 (2012).
44. Knight, W.E. *et al.* PDE1C deficiency antagonizes pathological cardiac remodeling and dysfunction. *Proc Natl Acad Sci U S A* **113**, E7116-E7125 (2016).
45. Wang, X. *et al.* Generation and phenotypic characterization of Pde1a mutant mice. *PLoS One* **12**, e0181087 (2017).
46. Wang, L. *et al.* Dual LQT1 and HCM phenotypes associated with tetrad heterozygous mutations in KCNQ1, MYH7, MYLK2, and TMEM70 genes in a three-generation Chinese family. *Europace* **18**, 602-9 (2016).
47. Vasti, C. & Hertig, C.M. Neuregulin-1/erbB activities with focus on the susceptibility of the heart to anthracyclines. *World J Cardiol* **6**, 653-62 (2014).
48. Xu, H. *et al.* A Genome-Wide Association Study of Idiopathic Dilated Cardiomyopathy in African Americans. *J Pers Med* **8**(2018).
49. Boczek, N.J. *et al.* Identification and Functional Characterization of a Novel CACNA1C-Mediated Cardiac Disorder Characterized by Prolonged QT Intervals With Hypertrophic Cardiomyopathy, Congenital Heart Defects, and Sudden Cardiac Death. *Circ Arrhythm Electrophysiol* **8**, 1122-32 (2015).
50. Kepenek, E.S. *et al.* Differential expression of genes participating in cardiomyocyte electrophysiological remodeling via membrane ionic mechanisms and Ca(2+)-handling in human heart failure. *Mol Cell Biochem* **463**, 33-44 (2020).
51. Mazzarotto, F. *et al.* Systematic large-scale assessment of the genetic architecture of left ventricular noncompaction reveals diverse etiologies. *Genet Med* **23**, 856-864 (2021).
52. Vasilescu, C. *et al.* Genetic Basis of Severe Childhood-Onset Cardiomyopathies. *J Am Coll Cardiol* **72**, 2324-2338 (2018).
53. Zhang, L. & Zhang, S. Learning common and specific patterns from data of multiple interrelated biological scenarios with matrix factorization. *Nucleic Acids Res* **47**, 6606-6617 (2019).
54. Prall, O.W. *et al.* An Nkx2-5/Bmp2/Smad1 negative feedback loop controls heart progenitor specification and proliferation. *Cell* **128**, 947-59 (2007).
55. Dorn, T. *et al.* Direct nkx2-5 transcriptional repression of isl1 controls cardiomyocyte subtype identity. *Stem Cells* **33**, 1113-29 (2015).
56. Sarcevic, B., Mawson, A., Baker, R.T. & Sutherland, R.L. Regulation of the ubiquitin-conjugating enzyme hHR6A by CDK-mediated phosphorylation. *EMBO J* **21**, 2009-18 (2002).

57. Roest, H.P. *et al.* The ubiquitin-conjugating DNA repair enzyme HR6A is a maternal factor essential for early embryonic development in mice. *Mol Cell Biol* **24**, 5485-95 (2004).
58. Roest, H.P. *et al.* Inactivation of the HR6B ubiquitin-conjugating DNA repair enzyme in mice causes male sterility associated with chromatin modification. *Cell* **86**, 799-810 (1996).
59. Lange, S., Pinotsis, N., Agarkova, I. & Ehler, E. The M-band: The underestimated part of the sarcomere. *Biochim Biophys Acta Mol Cell Res* **1867**, 118440 (2020).
60. Yang, J. *et al.* RBM24 is a major regulator of muscle-specific alternative splicing. *Dev Cell* **31**, 87-99 (2014).
61. Schneider, J.W. *et al.* Dysregulated ribonucleoprotein granules promote cardiomyopathy in RBM20 gene-edited pigs. *Nat Med* **26**, 1788-1800 (2020).
62. Sadeghi, L., Siggens, L., Svensson, J.P. & Ekwall, K. Centromeric histone H2B monoubiquitination promotes noncoding transcription and chromatin integrity. *Nat Struct Mol Biol* **21**, 236-43 (2014).
63. Young, M.D. *et al.* ChIP-seq analysis reveals distinct H3K27me3 profiles that correlate with transcriptional activity. *Nucleic Acids Res* **39**, 7415-27 (2011).
64. Liang, X. *et al.* HCN4 dynamically marks the first heart field and conduction system precursors. *Circ Res* **113**, 399-407 (2013).
65. Spater, D. *et al.* A HCN4+ cardiomyogenic progenitor derived from the first heart field and human pluripotent stem cells. *Nat Cell Biol* **15**, 1098-106 (2013).
66. Andres-Delgado, L. & Mercader, N. Interplay between cardiac function and heart development. *Biochim Biophys Acta* **1863**, 1707-16 (2016).
67. Ching, Y.H. *et al.* Mutation in myosin heavy chain 6 causes atrial septal defect. *Nat Genet* **37**, 423-8 (2005).
68. Hershberger, R.E. *et al.* Coding sequence rare variants identified in MYBPC3, MYH6, TPM1, TNNC1, and TNNI3 from 312 patients with familial or idiopathic dilated cardiomyopathy. *Circ Cardiovasc Genet* **3**, 155-61 (2010).

a

Figure 1: *Rnf20* is required for heart development

- a)** *Rnf20* mutant mouse survival chart for constitutive nulls, *Rnf20*^{flx/-}::*Nkx2.5*Cre⁺, and *Rnf20*^{flx/-}::*Isl1*Cre⁺. Colored percentages indicate the observed percentage of null or conditionally null mice (green indicates mendelian ratios, yellow indicates deviation from mendelian ratios, and red indicates no mice). Black percentages indicate the predicted percentages. The sample size is listed below each percentage. See Figure S2 for more details on these crosses. Images were taken of the cross between a *Nkx2.5*-cre positive (e8.5 (cardiac crescent), signal in cardiac crescent) or *Isl1*-cre positive mouse (e9.0 (heart tube), signal in outflow tract and atria) and a ROSA^{mt/mg} mouse to illustrate distribution of Cre-positive cells. Drawings indicate the expected morphology at each stage mouse heart development. OFT outflow tract, RV right ventricle, LV left ventricle, AVC atrioventricular canal, ECC endocardial cushions, RA right atrium, LA left atrium, A anterior, P posterior, L left, R - right.
- b)** Immunofluorescent staining for NKX2.5 and RNF20 in e11.5 wild-type (*Rnf20*^{fl/-}::*Nkx2.5*Cre⁻) and mutant (*Rnf20*^{fl/-}::*Nkx2.5*Cre⁺) mouse hearts.
- c)** Example hematoxylin and eosin stained e12.25 wild-type (*Rnf20*^{fl/-}::*Nkx2.5*Cre⁻) and mutant (*Rnf20*^{fl/-}::*Nkx2.5*Cre⁺) mouse. Quantifications of interventricular septum length, heart diameter, thickness of right ventricle compact myocardium, and thickness of left ventricle compact myocardium are displayed as individual data points with a line representing the median (n = 7 wild-type and 5 mutant hearts). Unpaired 2-tailed, heteroscedastic t-test.

Figure 2

Figure 2: H2Bub1 in iPSC-derived cardiomyocyte development shows selective maintenance of sarcomeric calcium genes

- a)** H2Bub1 surrounding the transcriptional start site (TSS) (-2 Kb, +10 Kb) in five stages of CM differentiation (iPSC, mesoderm (M), cardiac mesoderm (CMes), cardiac progenitor (CP), and cardiomyocyte (CM)). Genes are grouped by H2Bub1 occupancy into four clusters. The average profile, depicted using fold enrichment against random distribution values, across this region for each cluster in each stage is shown. This represents the average of three replicates.
- b)** The cluster breakdown for (a). Cluster 1 indicates high H2Bub1 levels, cluster 2 indicates low H2Bub1 levels, cluster 3 indicates moderate H2Bub1 levels, and cluster 4 indicates no H2Bub1.
- c)** Comparison of expressed transcripts from RNA-seq data (red) to the Ensembl transcripts near H2Bub1 peaks from ChIP-seq data (blue) at the CM stage.
- d)** Western blot for H2Bub1 in five stages of CM differentiation (iPSC, M, CMes, CP, and CM). The loading control for H2Bub1 is H2B. Numbers indicate average quantification (imageJ) of H2Bub1 normalized to H2B over two replicates.
- e)** Comparison of the number of Ensembl genes located near H2Bub1 peaks based on ChIP-seq data from CMes (blue) to CP (red).
- f)** Results of a differential binding analysis for H2Bub1-ChIP-seq across five stages of CM differentiation (iPSC, M, CMes, CP, and CM). This depicts the number of regions that gain and lose H2Bub1 at each transition.
- g)** A heat map depicting the top 20 significant regions with increased H2Bub1 occupancy in M compared with iPSCs.

759 **h)** The significant gene ontology terms from the genes near regions that maintain H2Bub1
760 between CMes and CP. Genes associated with the calcium signaling pathway are listed and
761 are colored blue if they are associated with cardiomyopathy from patient variants and/or
762 mouse models and are colored black if they are not.

763 **i)** Example H2Bub1 occupancy, depicted using fold enrichment against random distribution
764 across calcium genes (*CACNA1C* and *RYR2*, values ranging from 0 to 3) and non-calcium
765 genes (*KDM7A* and *NAT10*, values ranging from 0 to 8) at three stages in CM differentiation
766 (iPSC (blue), M (red), and CM (green)). All of the genes are oriented 5' to 3'. Gene structure
767 is indicated below the gene.

Figure 3

a

b

Figure 3: Reduced total H2Bub1 levels lead to abnormal cardiomyocytes

- a)** The percent of each 6 well plate of iPSC-derived cardiomyocytes that beats by day 20 is shown with colored dots (WT (n = 12) is blue, *RNF20*^{+/-} (mutant 1: n= 8, mutant 2: n = 11) is red, and *UBE2B*^{-/-} (mutant 1: n = 18, mutant 2: n = 11) is green). Data are shown as individual data points and a blue line representing the median. Unpaired 2-tailed, heteroscedastic t-test, * p<0.05, ** p < 0.01, N.S. is not significant.
- b)** Transmission electron microscopy of e12.25 wild-type (*Rnf20*^{fl/+}::*Nkx2.5*Cre⁺, n = 2) and mutant (*Rnf20*^{flx/-}::*Nkx2.5*Cre⁺, n = 2) mouse heart sarcomeres.

Figure 4

Figure 4: H2Bub1 level is determined by ratios of RNF20-complex components

- a)** RNA-seq data for RNF20-complex components (*RNF20*, *RNF40*, *UBE2B*, and *WAC*) in five stages of CM differentiation (iPSC, mesoderm (M), cardiac mesoderm (CMes), cardiac progenitor (CP), and cardiomyocyte (CM)) in wild-type cells. Data are shown as mean $\pm$ SEM (n = 3). Numbers indicate average ratio of *RNF20* to *WAC* mRNA over three replicates.
- b)** Western blot for RNF20-complex components (RNF20, RNF40, and UBE2B) in five stages of CM differentiation in wild-type cells. The loading control is GAPDH. The upper numbers indicate average quantification (imageJ) of the ratio of RNF20 normalized to GAPDH over UBE2B normalized to GAPDH for three replicates. The lower numbers indicate average quantification (imageJ) of the ratio of RNF20 normalized to GAPDH over RNF40 normalized to GAPDH for three replicates.
- c)** Quantified western blots (imageJ) for H2Bub1 normalized to H2B in five stages of CM differentiation in wild-type cells. Data are shown as the mean (n = 2).
- d)** Schematic depicting predicted ratio of the complex components (based on protein level for RNF20, RNF40 and UBE2B/A and mRNA level for WAC all determined by mean of three replicates) at the stage where H2Bub1 is the lowest (iPSC) and at the stage where H2Bub1 is the highest (CP) and the corresponding H2Bub1 levels. RNF20-red, RNF40-green, UBE2A/B blue, WAC-purple.
- e)** Western blot for RNF20-complex components (RNF20, RNF40, and UBE2B) in wild-type and *UBE2B*^{-/-} mutant iPSC and M cells. The loading control is GAPDH. The upper numbers indicate average quantification (imageJ) of the ratio of RNF20 normalized to GAPDH over UBE2B normalized to GAPDH for three replicates. The lower numbers indicate average

798 quantification (imageJ) of the ratio of RNF20 normalized to GAPDH over RNF40
799 normalized to GAPDH for three replicates.

800 **f)** Quantified western blots (imageJ) for H2Bub1 normalized to H2B in wild-type and *UBE2B*^{-/-}
801 mutant iPSC and M cells. Data are shown as the mean (n = 3).

802 **g)** Schematic depicting predicted ratio of the complex components (based on protein level for
803 RNF20, RNF40 and UBE2B/A all determined by mean of three replicates; WAC is assumed
804 based on WT data) at the iPSC stage in the *UBE2B*^{-/-} cells and the corresponding H2Bub1
805 level. RNF20-red, RNF40-green, WAC-purple.

806 **h)** RNA-seq data for RNF20-complex components (*RNF20*, *RNF40*, *UBE2B*, and *WAC*) in
807 wild-type and *RNF20*^{+/-}1 mutant iPSC and M cells. Data are shown as mean ± SEM (n = 3).
808 Numbers indicate average ratio of *RNF20* to *WAC* mRNA in the *RNF20*^{+/-}1 mutant cells over
809 three replicates.

810 **i)** Western blot for RNF20-complex components (RNF20, RNF40, and UBE2B) in wild-type and
811 *RNF20*^{+/-} mutant iPSC and M cells. The loading control is GAPDH. The upper numbers
812 indicate average quantification (imageJ) of the ratio of RNF20 normalized to GAPDH over
813 UBE2B normalized to GAPDH for three replicates. The lower numbers indicate average
814 quantification (imageJ) of the ratio of RNF20 normalized to GAPDH over RNF40
815 normalized to GAPDH for three replicates.

816 **j)** Quantified western blots (imageJ) for H2Bub1 normalized to H2B in wild-type and *RNF20*^{+/-}
817 mutant iPSC and M cells. Data are shown as the mean (n = 3).

818 **k)** Schematic depicting predicted ratio of the complex components (based on protein level for
819 RNF20, RNF40 and UBE2B/A and mRNA level for WAC all determined by mean of three

820 replicates) at the iPSC stage in the *RNF20*^{+/-} cells and the corresponding H2Bub1 level.
 821 RNF20-red, RNF40-green, UBE2A/B blue, WAC-purple.
 822 **l)** Western blot for RNF20-complex components (RNF20, RNF40, and UBE2B) in e12.25 wild-
 823 type (*Rnf20*^{fl/-}::*Nkx2.5Cre*⁻ and *Rnf20*^{fl/+}::*Nkx2.5Cre*⁺) and mutant (*Rnf20*^{fl/-}::*Nkx2.5Cre*⁺)
 824 mouse hearts. The loading control is alpha-tubulin. The upper numbers indicate
 825 quantification (imageJ) of the ratio of RNF20 normalized to alpha-tubulin over UBE2B
 826 normalized to alpha-tubulin. The lower numbers indicate quantification (imageJ) of the ratio
 827 of RNF20 normalized to alpha-tubulin over RNF40 normalized to alpha-tubulin.
 828 **m)** Quantified western blot (imageJ) for H2Bub1 normalized to H2B in e12.25 wild-type
 829 (*Rnf20*^{fl/-}::*Nkx2.5Cre*⁻ and *Rnf20*^{fl/+}::*Nkx2.5Cre*⁺) and mutant (*Rnf20*^{fl/-}::*Nkx2.5Cre*⁺) mouse
 830 hearts.
 831 **n)** Schematic depicting predicted ratio of the complex components (based on protein level for
 832 RNF20, RNF40 and UBE2B/A; WAC is assumed based on WT data) at e12.25 in *Rnf20*^{fl/-}
 833 ::*Nkx2.5Cre*⁺ and the corresponding H2Bub1 level. RNF40-green, UBE2A/B blue, WAC-
 834 purple.

Figure 5

a

b

c

d

Figure 5: Haploinsufficiency for *Rnf20* increases total H2Bub1 in iPSCs and arrests differentiation into cardiac mesoderm

- a) RNF20-complex schematic illustrating the *RNF20*^{+/-} iPSC mutant, and the associated increased total H2Bub1 levels.
- b) Genes with differing H2Bub1 occupancy (red) and gene expression (blue) when compared to wild-type that are shared between the two independent *RNF20*^{+/-} cell lines at the iPSC stage. Significant number of these genes overlap (Fisher's exact test $p = 3$).
- c) H2Bub1 surrounding the transcriptional start site (TSS) (-2 Kb, +10 Kb) in the wild-type and *RNF20*^{+/-} mutants at the iPSC stage. Genes are grouped by H2Bub1 levels into four clusters. The average profile, depicted using fold enrichment against random distribution values, across this region for each cluster in each stage is shown. This represents the average of three replicates.
- d) Gene ontology analysis of genes with differing H2Bub1 occupancy between wild-type and both independent *RNF20*^{+/-} cell lines at the iPSC stage. Many of these genes are related to transcription, splicing, and/or DNA/Protein modifications. Expression and H2Bub1 occupancy levels for example genes in each category are shown. Gene ontology (upper panel) is in gray scale; ChIP data (middle panel) and mRNA expression data (lower panel) are shown as heat maps.

Figure 6

a

b

c

Figure 6: Sarcomeric calcium signaling gene expression is reduced in cells with decreased total H2Bub1 levels

- a)** RNF20-complex schematic illustrating the *UBE2B*^{-/-} iPSC mutant and the *Rnf20*^{fl/-} mouse mutant. Both of these mutations lead to decreased total H2Bub1 levels. The mouse or cell icons in (b) and (c) indicate whether the analysis was done on mouse or cells.
- b)** Quantitative RT-PCR of calcium signaling genes in e11.5 wild-type (*Rnf20*^{fl/+}::*Nkx2.5Cre*⁺) and mutant (*Rnf20*^{fl/-}::*Nkx2.5Cre*⁺) mouse hearts for *Cacna1c*, *Ryr2*, *Ncx*, and *Serca2a*. Levels of expression are normalized to *18s* rRNA. Individual data points are shown in black dots. Data are shown as mean ± SEM (n = 2). Unpaired 1-tailed, heteroscedastic t-test, * p < 0.05, ** P < 0.01, N.S. is non-significant.
- c)** Gene ontology analysis on genes with differing gene expression levels comparing wild-type to both independent *UBE2B*^{-/-} cell lines at the cardiomyocyte stage. Many of these genes are related to sarcomere, cardiomyopathy, and/or calcium signaling. Expression levels for example genes in each category are shown. Stars indicate genes shared with maintained H2Bub1 marks upon transition to CPs shown in Fig. 2h. Data represent three RNA-seq replicates. Gene ontology (upper panel) is in gray scale; mRNA expression data (lower panel) is shown as a heat map.

Figure 7

Figure 7: Accumulation of H2Bub1 near the center of tissue specific genes is correlated with enhanced efficiency of transcriptional elongation

- a) Metagene plot for H2Bub1 levels in wild-type (blue) and *UBE2B*^{-/-} mutants (green and red) across the calcium signaling genes that are differentially expressed between wild-type and *UBE2B*^{-/-} mutants and/or selectively maintained between cardiac mesoderm and cardiac progenitor stages (n = 3). The * indicates the accumulation near the center of the gene.
- b) Metagene plot for H2Bub1 levels in wild-type (blue) and *UBE2B*^{-/-} mutants (green and red) across the sarcomeric genes that are differentially expressed between wild-type and *UBE2B*^{-/-} mutants (n = 3). The * indicates the accumulation near the center of the gene.
- c) Example metagene plot for H2Bub1 levels in wild-type (blue) and *UBE2B*^{-/-} mutants (green and red) across “randomly” selected quantity and size-matched genes to the calcium gene set (n = 3). 30 “random” metagene plots were created from the calcium gene set and 30 “random” metagene plots were created from the sarcomere gene set: 10 from genes that are upregulated between wild-type and both *UBE2B*^{-/-} cell lines, 10 that are non-regulated between wild-type and both *UBE2B*^{-/-} cell lines, and 10 that are down-regulated between wild-type and both *UBE2B*^{-/-} cell lines.
- d) Log2 of fold change in transcript abundance between wild-type and *UBE2B*^{-/-} mutants is shown at each position along the genes. The genes being shown are the calcium signaling genes describe in (a).
- e) Log2 of fold change in transcript abundance between wild-type and *UBE2B*^{-/-} mutants is shown at each position along the genes. The genes being shown are the sarcomeric genes described in (b).

- f)** Log2 of fold change in transcript abundance between wild-type and *UBE2B*^{-/-} mutants is shown at each position along the genes. The genes being shown are “randomly” selected quantity and size-matched genes to the calcium signaling genes described in (c).
- g)** Example log2 of fold change gene traces for calcium genes (*CACNA1C* and *RYR2*) that have accumulation of H2Bub1 near the center of the gene and decreased transcriptional elongation efficiency. 5 on the left of the diagram.
- h)** Metagene plot for H2Bub1 levels in wild-type MEFs (teal) and wild-type mESCs (red) across the ECM genes that are differentially expressed between wild-type MEFs and wild-type mESCs. The * indicates the accumulation near the center of the gene.
- i)** Metagene plot for H2Bub1 levels in wild-type MEFs (teal) and wild-type mESCs (red) across the adhesion genes that are differentially expressed between wild-type MEFs and wild-type mESCs. The * indicates the accumulation near the center of the gene.
- j)** Example metagene plot for H2Bub1 levels in wild-type MEFs (teal) and wild-type mESCs (red) across “randomly” selected quantity and size-matched genes to the adhesion gene set. 20 “random” metagene plots were created from the adhesion gene set and 20 “random” metagene plots were created from the ECM gene set.

Figure 8

a

b

c

Figure 8: Working Model

- a) When H2Bub1 levels are misregulated by altering levels of complex components, cardiomyocytes do not form normally. When total H2Bub1 levels are increased by haploinsufficiency of RNF20, cardiomyocytes do not form. When total H2Bub1 levels are decreased by creating *UBE2B*^{-/-} or *Rnf20*^{fl/-}, sarcomeres do not form normally and calcium signaling genes have reduced expression.
- b) During wild-type CM differentiation, housekeeping genes and heterochromatic regions have dynamic H2Bub1 levels. H2Bub1 is sparsely maintained during the transition from cardiac mesoderm to cardiac progenitor. Notably, selectively maintained genes are enriched for sarcomeric calcium genes.
- c) Genes that are short in length have an increase in H2Bub1 at the 5' end of the gene that decreases towards the 3' end. However, longer tissue-specific genes have an accumulation in H2Bub1 near the center of the gene in wild-type cells and not in *UBE2B*^{-/-}. In the mutants when this accumulation is absent, the transcriptional efficiency is reduced.

Supplementary Figure 1

Supplementary Figure 1: RNF20 is dynamically expressed during mouse heart development

a) Immunofluorescent staining for RNF20 in different sections (atrium, anterior ventricle, and posterior ventricle) of the e9.5 wild-type mouse heart.

b) Immunofluorescent staining for RNF20 and WT1 (epicardial marker) in e11.5, e16.5, and P0 wild-type mouse hearts. These stages capture the major changes in RNF20 expression in the epicardial cells.

c) Immunofluorescence staining for RNF20 in the myocardial cells in e11.5, e14.5, and e16.5 wild-type mouse hearts. These stages capture the major changes in RNF20 expression in the myocardial cells.

d) Immunofluorescence staining for RNF20 and CD31 (endocardial marker) in e11.5, e14.5, and P0 wild-type mouse hearts. These stages capture the major changes in RNF20 expression in the endocardial cells.

e) Western blot for RNF20-complex components (RNF20, RNF40, and UBE2B) and H2Bub1 in wild-type mouse hearts (e9.5, e11.5, e14.5, e16.5, P0, P224 female, and P224 male). The loading control for the complex components is total protein (shown to the right of the western blots) and for H2Bub1 is H2B.

Supplementary Figure 2

Supplementary Figure 2: Breeding strategy and phenotypes of *Rnf20* mutants

- a)** Diagram illustrating the *Rnf20* deletion construct. This mouse was created by knocking out exons 2-19 (exon 1 is non-coding and exon 20 is mostly non-coding) and replacing them with a LacZ neo cassette. The neo cassette was dropped using the LoxP sites.
- b)** Percent of each genotype of P0 mice born from the cross of two *Rnf20*^{+/-} mice.
- c)** Diagram illustrating the mouse breeding scheme for the conditional knock-out mice.
- d)** Percent of each genotype of e9.5, e12.5, P0, and P224 from the cross of *Rnf20*^{fl/fl} and *Rnf20*^{+/-} ::*Isl1*Cre⁺.
- e)** Heart weight/body weight ratios for 14 (6 *Rnf20*^{fl/-} ::*Isl1*Cre⁻, 3 *Rnf20*^{fl/+} ::*Isl1*Cre⁺, 3 *Rnf20*^{fl/+} ::*Isl1*Cre⁻, and 2 *Rnf20*^{fl/-} ::*Isl1*Cre⁺) adult offspring (P224) from the cross of *Rnf20*^{fl/fl} and *Rnf20*^{+/-} ::*Isl1*Cre⁺. Data are shown as mean ± SEM.
- f)** Example hematoxylin and eosin stained P224 wild-type (*Rnf20*^{fl/-} ::*Isl1*Cre⁻) and mutant (*Rnf20*^{fl/-} ::*Isl1*Cre⁺) mouse hearts.
- g)** Example hematoxylin and eosin stained e11.5 wild-type (*Rnf20*^{fl/-} ::*Nkx2.5*Cre⁻) and mutant (*Rnf20*^{fl/-} ::*Nkx2.5*Cre⁺). Lower panels are higher magnification views of the ventricles.
- h)** Percent of each genotype of e9.5, e11.5, e12.25, e12.5, and P0 from the cross of *Rnf20*^{fl/fl} and *Rnf20*^{+/-} ::*Nkx2.5*Cre⁺.

Supplementary Figure 3

Supplementary Figure 3: H2Bub1 and mRNA expression throughout CM differentiation

- a)** PCA plots of RNA-seq experiments of CM differentiation comparing all neighboring stages (iPSC (red) v mesoderm (M) (blue), M (red) v cardiac mesoderm (CMes) (blue), CMes (red) v cardiac progenitor (CP) (blue), and CP (red) v cardiomyocyte (CM) (blue)) for three replicates of each stage.
- b)** The top 30 gene ontology (GO) terms related to development in cluster 2 (see Fig. 2a, 2b) of iPSCs. Dashed bars represent GO terms not related to heart, nervous system, or appendages.
- c)** The top 30 GO terms related to development in cluster 2 (see Fig. 2a, 2b) of CMs.
- d)** One additional replicate of western blots (see Figure 2d) showing H2Bub1 changes during five stages of CM differentiation (iPSC, M, CMes, CP, and CM). The loading control is H2B.
- e)** Percent of H2Bub1 peaks overlapping with the B compartment in each stage of CM differentiation (iPSC, M, CP, and CM) comparing observed (red) and expected (blue) values. Data are shown as mean $\pm$ SEM (n = 3). P-values comparing observed and expected values were manually calculated by comparing the actual values to the expected values, * p < 0.05, ** P < 0.01 (**online methods**). P-values comparing observed values between stages were calculated by computing a z score, * p < 0.05, ** P < 0.01.
- f)** Percent of H2Bub1 peaks on the same gene as an activating chromatin mark (H3K4me3 shown in blue) and a repressive chromatin mark (H3K27me3 shown in red) in iPSCs and CMs. Data are the ratio of observed to expected values. Data are shown as mean $\pm$ SEM (n = 3). P-values comparing observed values to expected values were calculated by Chi Square tests and adjusted for multiple comparisons, * p < 0.05, ** P < 0.01. P-values comparing

978 observed values between stages were calculated using an unpaired 2-tailed, heteroscedastic t-
979 test, * $p < 0.05$, ** $P < 0.01$, N.S. is non-significant.

Supplementary Figure 4

Supplementary Figure 4: Localized regions of H2Bub1 in the cardiac mesoderm stage predict decreased expression in the cardiac progenitor stage

a) Patterns identified in the H2Bub1 peaks in cardiac mesoderm (CMes) and in cardiac progenitor (CP). The graphs depict the H2Bub1 occupancy in the clusters of genes, called patterns, over the gene body. Heat maps showing patterns based on amount are in the left panel, and based on distribution are shown in the right panel. A pattern is defined as a group of genes that have a similar structure to the distribution or amount of H2Bub1 across the gene body.

b) Ratio of H2Bub1 regions lost to total H2Bub1 regions in each of the patterns (based on amount in the left panel and based on distribution in the right panel) and the average ratio (labeled all) between CMes and CP.

c) Expression levels from the RNA-seq data of the genes in each pattern (based on amount in the left panel and based on distribution in the right panel) in CMes (red) and CP stages (blue). Data are shown as a box plot, where the center line is the median, the box limits are the upper and lower quartiles, and the whiskers are 1.5x the interquartile range. Paired, one-sided Wilcoxon Rank Sum Test, * $p < 0.05$, ** $p < 0.01$.

Supplementary Figure 5

Supplementary Figure 5: Characteristics of the *RNF20*^{+/-} mutants

- a)** Diagram of the RNF20 protein with its domains (Ring-Finger domain is shown in blue) and where the created *RNF20* mutations are located (with a star). Lower four panels are sequencing traces of the wild-type and two independent *RNF20*^{+/-} iPSC lines demonstrating CRISPR-generated mutations.
- b)** PCA plots of RNA-seq experiments comparing wild-type (red) and both *RNF20*^{+/-} mutants (green and blue) at iPSC and mesoderm (M) stages (n = 3).
- c)** H2Bub1 surrounding the transcriptional start site (TSS) (-2 Kb, +10 Kb) in the wild-type and *RNF20*^{+/-} mutants at the M stage. Genes are grouped by H2Bub1 levels into four clusters. The average profile, depicted using fold enrichment against random distribution values, across this region for each cluster in each stage is shown. This represents the average of three replicates.
- d)** Regions with differential H2Bub1 in each *RNF20*^{+/-} mutant compared to wild-type at the M stage. These Venn Diagrams depict the overlap between the regions that have increased H2Bub1 in *RNF20*^{+/-} mutants compared to wild-type and the regions that have decreased H2Bub1 in both *RNF20*^{+/-} mutants compared to wild-type. *RNF20*^{+/-} line 1 is in blue, *RNF20*^{+/-} line 2 is in red.

Supplementary Figure 6

1013 **Supplementary Figure 6: Analysis of RNF20-complex components and H2Bub1**

- 1014 **a)** Two additional replicates of western blots (see Figure 4b) showing RNF20-complex
1015 components changing during CM differentiation. Western blot for RNF20, RNF40, and
1016 UBE2B in five stages of CM differentiation (iPSC, mesoderm (M), cardiac mesoderm
1017 (CMes), cardiac progenitor (CP), and cardiomyocyte (CM)). The loading control is GAPDH.
- 1018 **b)** Two additional western blot replicates (see Figure 4e) depicting RNF20-complex components
1019 (RNF20, RNF40, and UBE2B) in iPSC and M stages comparing wild-type and *UBE2B*^{-/-}
1020 cells. The loading control is GAPDH.
- 1021 **c)** Three western blot replicates (See Figure 4f for the quantification) depicting H2Bub1 in iPSC
1022 and M stages comparing wild-type and *UBE2B*^{-/-} cells. The loading control is H2B.
- 1023 **d)** Two additional western blot replicates (see Figure 4i) depicting RNF20-complex components
1024 (RNF20, RNF40, and UBE2B) in iPSC and M stages comparing wild-type and *RNF20*^{+/-}
1025 cells. The loading control is GAPDH.
- 1026 **e)** Three western blot replicates (See Figure 4j for the quantification) depicting H2Bub1 in iPSC
1027 and M stages comparing wild-type and *RNF20*^{+/-} cells. The loading control is H2B.
- 1028 **f)** RNA-seq data for RNF20-complex components (*RNF20*, *RNF40*, *UBE2B*, and *WAC*) in wild-
1029 type and *RNF20*^{+/-2} mutant iPSC and M cells. Data are shown as mean ± SEM (n = 3).
- 1030 **g)** One western blot replicate (See Figure 4m for the quantification) depicting H2Bub1 in e12.25
1031 wild-type (*Rnf20*^{fl/-}::*Nkx2.5Cre*⁻ and *Rnf20*^{fl/+}::*Nkx2.5Cre*⁺) and mutant (*Rnf20*^{fl/-}
1032 ::*Nkx2.5Cre*⁺) mouse hearts. The loading control is H2B.

1033 **Supplementary Figure 7: Differentiation trajectory for RN20 complex mutants**

1034 **a)** Immunofluorescence staining for marker genes for each of five stages in CM differentiation

1035 (iPSC: OCT4 (red), mesoderm (M): Brachyury (green), cardiac mesoderm (CMes): NKX2.5
1036 (red), cardiac progenitor (CP): ISL1 (green), and cardiomyocyte (CM): TNNT (green)) in
1037 each of the mutants (wild-type, *RNF20*^{+/-}1, *RNF20*^{+/-}2, *UBE2B*^{-/-}1, and *UBE2B*^{-/-}2) at the
1038 iPSC stage.

1039 **b)** Immunofluorescence staining for marker genes for each of five stages in CM differentiation

1040 (iPSC: OCT4 (red), M: Brachyury (green), CMes: NKX2.5 (red), CP: ISL1 (green), and CM:
1041 TNNT (green)) in each of the mutants (wild-type, *RNF20*^{+/-}1, *RNF20*^{+/-}2, *UBE2B*^{-/-}1, and
1042 *UBE2B*^{-/-}2) at the M stage.

1043 **c)** Immunofluorescence staining for marker genes for each of five stages in CM differentiation

1044 (iPSC: OCT4 (red), M: Brachyury (green), CMes: NKX2.5 (red), CP: ISL1 (green), and CM:
1045 TNNT (green)) in each of the mutants (wild-type, *RNF20*^{+/-}1, *RNF20*^{+/-}2, *UBE2B*^{-/-}1, and
1046 *UBE2B*^{-/-}2) at the CM stage. Since these mutants form beating CMs some of the time,
1047 staining was done for beating and non-beating samples for each genotype, except *RNF20*^{+/-}2
1048 since it never forms beating CMs.

Supplementary Figure 8

1049 **Supplementary Figure 8: Characteristics of the *UBE2B*^{-/-} mutant**

- 1050 **a)** Diagram of the UBE2B protein with its catalytic site, shown in blue, and where the created
1051 *UBE2B* mutations are located (with a star). Lower three panels are sequencing traces of the
1052 wild-type and two independent *UBE2B*^{-/-} iPSC lines demonstrating CRISPR-generated
1053 mutations.
- 1054 **b)** PCA plots of RNA-seq experiments comparing wild-type (red) and both *UBE2B*^{-/-} mutants
1055 (green and blue) at the cardiomyocyte stage (n = 3).
- 1056 **c)** H2Bub1 surrounding the transcriptional start site (TSS) (-2 Kb, +10 Kb) in the wild-type and
1057 *UBE2B*^{-/-} mutants at the cardiomyocyte stage. Genes are grouped by H2Bub1 levels into four
1058 clusters. The average profile, depicted using fold enrichment against random distribution
1059 values, across this region for each cluster in each stage is shown. This represents the average
1060 of three replicates.
- 1061 **d)** Metagene plot for H2Bub1 levels in wild-type (blue) and *UBE2B*^{-/-} mutants (green and red)
1062 across all of the short genes (less than 100 bp) (n = 3).
- 1063 **e)** Metagene plot for H2Bub1 levels in wild-type (blue) and *UBE2B*^{-/-} mutants (green and red)
1064 across all of the long genes (greater than 10 Kb) (n = 3). The * indicates the accumulation
1065 near the center of the gene.

1066 **Supplemental Table 1: Primers** A list of all of the primers used in this study and their
 1067 application.

Primer name	Sequence	Species	Application
<i>I8s</i>	CTT C T C	Mouse	Calcium RT-PCR experiment
<i>I8s</i> R	C CT CC TC T T	Mouse	Calcium RT-PCR experiment
<i>Cacna1c</i>	CCT T TTC TTTC T	Mouse	Calcium RT-PCR experiment
<i>Cacna1c</i> R	TCC TT CCTCC TC	Mouse	Calcium RT-PCR experiment
<i>Ncx</i>	TC C TCT C T T	Mouse	Calcium RT-PCR experiment
<i>Ncx</i> R	CTCC C CTCC C	Mouse	Calcium RT-PCR experiment
<i>Ryr2</i>	CCTT CCT T C TT	Mouse	Calcium RT-PCR experiment
<i>Ryr2</i> R	TT T TC C TT T	Mouse	Calcium RT-PCR experiment
<i>Serca2a</i>	C T T TC C	Mouse	Calcium RT-PCR experiment
<i>Serca2a</i> R	TC C CTTT TC CC	Mouse	Calcium RT-PCR experiment
<i>GAPDH</i> exon	C CC T T TTC	human	Control or 2 u 1 ChIP-se
<i>GAPDH</i> exon R	C T C CCT T	human	Control or 2 u 1 ChIP-se
<i>GAPDH</i> Promoter	CTCTCC C TC TC	human	Control or 2 u 1 ChIP-se
<i>GAPDH</i> Promoter R	C T CTTCCC TTC	human	Control or 2 u 1 ChIP-se
<i>RNF20</i>	C CT TCTTCTTCT CC	human	enot ping iPSC Mutants
<i>RNF20</i> R	CC CCTTCCC TTCTC	human	enot ping iPSC Mutants
<i>RNF20</i> Sequence	CTTCCCC T CTCC	human	enot ping iPSC Mutants
<i>UBE2B</i>	C TC TT C TT TTT	human	enot ping iPSC Mutants
<i>UBE2B</i> R	C C CTT T T	human	enot ping iPSC Mutants
<i>UBE2B</i> Sequence	CCCTT CC CTT TC	human	enot ping iPSC Mutants
<i>Rnf20</i> ^{+/-} null allele	CC TT CC TT TCT T TC	Mouse	Mouse genot ping
<i>Rnf20</i> ^{+/-} T allele	CCTTC CCTC TCT C	Mouse	Mouse genot ping
<i>Rnf20</i> ^{+/-} R null allele	TCCTC CT T TTCTCTC CT CT	Mouse	Mouse genot ping
<i>Rnf20</i> ^{+/-} R T allele	TCCTC CT T TTCTCTC CT CT	Mouse	Mouse genot ping
<i>UBE2B</i>	C CT C C T TC	human	Se uencing c
<i>UBE2B</i> R	C C T CC CTCTT	human	Se uencing c

1068

1069 **Supplemental Table 2: Antibodies** A list of all of the antibodies used in this study, their
 1070 species, their dilution, and their application.

Antibody	Catalogue number	Species	Dilution	Application
2 u 1	Cell Signaling 55 s	Rat		ChIP
Igg	Cell Signaling 2 2 s	Rat		ChIP
Archur	R s stems 2085-SP	oat	1 20	I cells
Cardiac Troponin T	Invitrogen M 5-12 0	Mouse	1 200	I cells
IS 1	S 3 . 5 supernatant	Rat	1 10	I cells
2.5	Santa Cru sc-8	oat	1 200	I cells
CT- a	Cell Signaling 28 0S	Rat	1 200	I cells
C 31	iosciences 5502	Rat	1 500	I sections
2.5	Santa Cru sc-8	oat	1 100	I sections
R 20	Cell Signaling 11	Rat	1 200	I sections
T1	ovus -h2	Mouse	1 250	I sections
alpha-tu ulin	Invitrogen 220	Mouse	1 1000	estern
P	Invitrogen M 515 38 RP	Mouse	1 20 000	estern
2	Millipore 0 -3 1	Rat	1 12 000	estern
2 u 1	Cell Signaling 55 s	Rat	1 200 000	estern
R 20	Cell Signaling 11	Rat	1 500	estern
R 0	cam a 1 130	Rat	1 1000	estern
U 2	enTex T 100 1	Rat	1 500	estern

1071

1072 **Movie Legends**

1073 **Movie 1:** Movie shows wild-type cardiomyocytes under bright-field microscopy.

1074

1075 **Movie 2:** Movie shows *RNF20^{+/-}1* cardiomyocytes beating under bright-field microscopy.

1076

1077 **Movie 3:** Movie shows *RNF20^{+/-}1* cardiomyocytes not beating under bright-field microscopy.

1078

1079 **Movie 4:** Movie shows *RNF20^{+/-}2* cardiomyocytes not beating under bright-field microscopy.

1080

1081 **Movie 5:** Movie shows *UBE2B^{-/-}1* cardiomyocytes beating under bright-field microscopy.

1082

1083 **Movie 6:** Movie shows *UBE2B^{-/-}1* cardiomyocytes not beating under bright-field microscopy.

1084

1085 **Movie 7:** Movie shows *UBE2B^{-/-}2* cardiomyocytes beating under bright-field microscopy.

1086

1087 **Movie 8:** Movie shows *UBE2B^{-/-}2* cardiomyocytes not beating under bright-field microscopy.

1088 **Supplemental Data 1: ChIP-seq wild-type iPSC-derived cardiomyocytes**

1089 **Tab 1:** contains the genes in Clusters 1-4 displayed in Fig. 2a and Fig. 5c.

1090 **Tabs 2-21:** contain the gene ontology terms for the genes in Clusters 1-4 in iPSC, mesoderm
1091 (M), cardiac mesoderm (CMes), cardiac progenitor (CP), and cardiomyocyte (CM) in Fig. 2a.

1092 **Tabs 22-29:** contain the differentially occupied regions between all of the consecutive stages
1093 (iPSC v. M, M v. CMes, CMes v. CP, and CP v. CM) of CM differentiation (Fig. 2).

1094 **Tabs 30-31:** contain the genes in each pattern of each version (Fig. S4).

1095

1096 **Supplemental Data 2: RNA-seq wild-type iPSC-derived cardiomyocytes**

1097 **Tabs 1-8:** contain the genes that are differentially expressed in between all of the consecutive
1098 stages (iPSC v. mesoderm (M), M v. cardiac mesoderm (CMes), CMes v. cardiac progenitor
1099 (CP), and CP v. cardiomyocyte (CM)) of CM differentiation (Fig. S3).

1100 **Tab 9:** contains the RNA-seq quality metrics.

1101

1102 **Supplemental Data 3: ChIP-seq *RNF20*^{+/-} iPSCs and mesoderm**

1103 **Tabs 1-4:** contain the differentially occupied regions between wild-type and *RNF20*^{+/-} iPSCs
1104 (Fig. 5).

1105 **Tab 5:** contains the genes in Clusters 1-4 displayed in Fig. S5c.

1106 **Tabs 6-9:** contain the differentially occupied regions between wild-type and *RNF20*^{+/-} mesoderm
1107 (Fig. S5).

1108

1109

1110

1111 **Supplemental Data 4: RNA-seq *RNF20*^{+/-} iPSCs and mesoderm**

1112 **Tabs 1-8:** contain the genes that are differentially expressed in between wild-type and *RNF20*^{+/-}

1113 iPSCs, and wild-type and *RNF20*^{+/-} mesoderm (Fig. 5, S5).

1114 **Tab 9:** contains the RNA-seq quality metrics.

1115

1116 **Supplemental Data 5: ChIP-seq *UBE2B*^{-/-} cardiomyocytes**

1117 **Tab 1:** contains the genes in Clusters 1-4 displayed in Fig. S8c.

1118 **Tabs 2-5:** contain the differentially occupied regions between wild-type and *UBE2B*^{-/-}

1119 cardiomyocytes (Fig. S8).

1120 **Tabs 6-10:** contain the genes used for each metagene plot (Fig. 7).

1121

1122 **Supplemental Data 6: RNA-seq *UBE2B*^{-/-} cardiomyocytes**

1123 **Tabs 1-4:** contain the genes that are differentially expressed in between wild-type and *UBE2B*^{-/-}

1124 cardiomyocytes (Fig. 6, S8).

1125 **Tab 5:** contains the RNA-seq quality metrics.

1126 **Online Methods**

1127 **Mouse husbandry**

1128 The wild-type mouse data in Figure S1 are on the CD1 background. The *Rnf20*^{+/-}, *Rnf20*^{fl/fl}, and
1129 all of the cre lines used in this study are maintained on the C57BL/6 background. All cre mice
1130 used were validated for correct expression and penetrance by testing their offspring with a
1131 ROSA^{mT/mG} mouse at e8.5 for GFP and tdTomato expression. This research complies with all
1132 ethical regulations; mouse experiments were performed in a manner approved by the Yale
1133 University Institutional Animal Care and Use Committee.

1134

1135 **Cell lines and cardiomyocyte differentiation**

1136 PGP1 cells (gift from the Seidman lab) were maintained on matrigel with mTESR media.
1137 Differentiation was performed in accordance with previously published work³⁷. Briefly, 0.5
1138 million cells (or 0.7 million cells in the *UBE2B*^{-/-} mutants), were plated on matrigel about four
1139 days prior to onset of differentiation. When the cells reached 100% confluency, differentiation
1140 was started using 12 μ M of CHIR99021 in RPMI media supplemented with B-27 minus insulin.
1141 Twenty-four hours later, the media was replaced with RPMI media supplemented with B-27
1142 minus insulin. Forty-eight hours later, the media was replaced with 5 μ M of IWP4 in RPMI
1143 media supplemented with B-27 minus insulin. Forty-eight hours later, the media was replaced
1144 with RPMI media supplemented with B-27 minus insulin. On day eight and every 2-3 days after
1145 that, the media was replaced with RPMI media supplemented with B-27. After the onset of
1146 beating, the cardiomyocytes (CMs) were selected using 4 mM lactate in DMEM without glucose.
1147 A well was considered to be beating if two distinct regions on the plate contained beating cells.

1148 Prior to the collection of samples, mycoplasma testing was performed on all cell lines using the
1149 MycoAlert Plus Mycoplasma Detection Kit from Lonza (LT07-703).

1150

1151 Since lactate selection is a metabolic selection, it can only work if the cells are CMs. Therefore,
1152 if a well was not beating, no lactate selection was done and the subsequent analyses were done
1153 on the non-selected cells.

1154

1155 **CRISPR-Cas9 editing of human iPSCs**

1156 guides were created using MIT's CRISPR tool (<http://crispr.mit.edu/>) to create mutations in
1157 *RNF20* and *UBE2B* (2 each) near the transcriptional start site. These guides and the other
1158 necessary sequence were synthesized as gblocks and cloned using the TOPO TA cloning kit.
1159 Two different protocols were used to create knockouts. *UBE2B*^{-/-}, *RNF20*^{+/-}, and *RNF20*^{+/-}2
1160 were created using the Lonza Nucleofector 2b with the Human Stem Cell Nucleofector Kit 2
1161 according to the manufacturer's protocol. 1 million cells were nucleofected using program B020
1162 with 2 µg of a plasmid containing Cas9 (PX459 Addgene) and 2 µg of a plasmid containing the
1163 sgRNA. *UBE2B*^{-/-}2 was created by adding 10 µg of Cas9 plasmid and 10 µg of sgRNA plasmid
1164 to 1 million cells using the Bio-Rad Gene Pulser XCell Eukaryotic System with a homemade
1165 nucleofection solution (5 mM KCl, 5 mM MgCl₂, 15 mM Hepes, 102.94 mM Na₂HPO₄, 47.06
1166 mM NaH₂PO₄ monohydrate, pH = 7.2) according to previously published work. The following
1167 parameters were used: 250 V, 500 µF, and a 0.4 cm cuvette⁶⁸. These cells grew for two days with
1168 mTESR supplemented with 5 µM of Y27632. Then, selection for receiving the Cas9 plasmid
1169 occurred for 2 days using 400 µg of puromycin. About a week after transfection, individual
1170 colonies were picked and screened for mutations using primers flanking the sgRNA

1171 **(Supplemental Table 1).** If there was a possibility of a mixed colony, individual colonies got
 1172 picked from that colony and these subcolonies got screened. This process was repeated until
 1173 colonies were pure. Additionally, the DNA was cloned into TOPO TA cloning it (*RNF20*) or
 1174 Zero Blunt TOPO Cloning Kit (*UBE2B*) and sequenced to verify the ratio of the two different
 1175 alleles was 1:1.

1176

Guide	Sequence	Cell Line(s) Developed
RNF20_1	ACAGTGGAAACAATTAAGCT	<i>RNF20</i> ^{+/-} 1 and <i>RNF20</i> ^{+/-} 2
RNF20_2	GTGGAAACAATTAAGCTAGG	N/A
UBE2B_1	AATCCCGCATGAGCCTCCTC	<i>UBE2B</i> ^{-/-} 1
UBE2B_2	ATCCCGCATGAGCCTCCTCC	<i>UBE2B</i> ^{-/-} 2

1177

1178 *Surrounding sequence to the gRNA*

1179 TGTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACTGGATCCGGTACCAA
 1180 GGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATA
 1181 CAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACACAAAGATATTAGT
 1182 ACAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAATT
 1183 ATGTTTTAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTCGATTTCTTG
 1184 GCTTTATATATCTTGTGGAAAGGACGAAACACC (Guide)
 1185 GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAA
 1186 AGTGGCACCGAGTCGGTGCTTTTTTTCTAGACCCAGCTTTCTTGTACAAAGTTGGCAT
 1187 TA

1188

1189 **Immunofluorescence of mice**

1190 Mouse embryos and hearts were fixed in 4% PFA overnight. They were placed in 30% sucrose
 1191 until the tissue sank and then embedded in OCT. 12 μ M sections were cut. These slides were

allowed to defrost and dry out for 3 hours on a slide warmer. The sections were post-fixed for 10 minutes with 0.2% PFA, and then washed 4 times with PBS for 20 minutes. The sections were either blocked in 5% BSA, 10% Goat serum, or Fish Gelatin Blocking agent in PBT (PBS+ 0.2% Triton X100). The samples were incubated with primary antibodies for either 4 hours at RT or overnight (**Supplemental Table 2**). The samples were washed four times in PBT for about 20 minutes, incubated with the corresponding chromophore conjugated secondary antibody (1:500) for about 45 minutes, and then washed four times in PBT for about 20 minutes. The second to last wash contained 1:2000 Hoechst. The samples were mounted on coverslips in ProLong Gold (molecular probes). The samples were imaged on the Zeiss Observer Z1 equipped with Apotome optical interference imaging.

Immunofluorescence of cells

iPSCs and mesodermal (M) cells were passaged using accutase. 650 μ L of accutase was added to each well of a 6 well plate for 5-7 minutes at 37°C until the cells started to come off from the plate. After spinning down at 1000 rpm for 5 minutes, the cells were plated on coverslips. A 0.1% w/v solution of gelatin had been added to these coverslips overnight at 37°C. The next morning, these coverslips were rinsed with PBS, a 1/400 solution of matrigel in media was added for 1 hour, rinsed with PBS, and then the passaged cells were added. The media the cells were grown in while they were on the coverslips was normal cell culture media for the respective stage with 5 μ M of Y27632.

CMs were passaged using Trypsin-EDTA (0.25%). 2 mL of Trypsin was added to each well of a 6 well plate for 5 minutes at room temperature. After spinning down at 1000 rpm for 5 minutes,

1215 the cells were plated on coverslips. A 0.1% w/v solution of gelatin had been added to these
1216 coverslips overnight at 37°C. The next morning, these coverslips were rinsed with PBS, and then
1217 the passaged cells were added. These cells were grown in RPMI20 (RPMI with 20% FBS) with 5
1218 μ M of Y27632 as per previous work³⁷.

1219

1220 After 2 days of culturing the iPSC, M, or CMs on coverslips, they were fixed in 4% PFA for 10
1221 minutes. Extreme caution was taken when adding and removing liquids to not cause the cells to
1222 come off the coverslips. The coverslips were rinsed 4 times for 20 minutes with PBS. The
1223 sections were either blocked in 5% BSA or 10% Goat serum in PBT (PBS+ 0.2% Triton X100).
1224 The samples were incubated with primary antibodies for either 4 hours at RT or overnight
1225 (**Supplemental Table 2**). The samples were washed four times in PBT for about 20 minutes,
1226 incubated with the corresponding chromophore conjugated secondary antibody (1:500) for about
1227 45 minutes, and then washed four times in PBT for about 20 minutes. The second to last wash
1228 contained 1:2000 Hoechst. The coverslips were mounted on slides in ProLong Gold (molecular
1229 probes). The samples were imaged on the Zeiss Observer Z1 equipped with Apotome optical
1230 interference imaging.

1231

1232 **Histology**

1233 Whole embryos or adult hearts were fixed overnight in 4% buffered paraformaldehyde. The adult
1234 hearts were then cut in half sagittal sections. These embryos or half hearts were dehydrated,
1235 cleared, and embedded in paraffin wax. Transverse sections (embryonic sections were 10 μ m
1236 thick and adult heart sections were 3-6 μ m thick) were cut using a microtome and were stained
1237 with hematoxylin and eosin following standard protocols. Images were taken on a Zeiss Axiovert

1238 microscope, using an Axiocam driven by Axiovision software. Measurements (heart size,
1239 interventricular septum length, and compact myocardium thickness) were made using imageJ⁶⁹.

1240

1241 **Transmission electron microscopy**

1242 The embryos were dissected in 4% PFA. A small hole was made in an atrium with a scalpel to
1243 allow the fixative to get inside the heart. Embryonic heart tissue was fixed in 2.5%
1244 glutaraldehyde and 2% paraformaldehyde in 0.1M sodium cacodylate buffer, pH 7.4 for 1 hour
1245 at room temperature and left at 4°C for two days. The samples were rinsed in buffer, then post
1246 fixed in 1% osmium tetroxide and en-bloc stained in 2% aqueous uranyl acetate for another hour.
1247 This was followed by rinsing in distilled water and dehydrating in an ethanol series, then resin
1248 infiltration in Embed 812 (Electron Microscopy Sciences) and baking overnight at 60°C.
1249 Hardened blocks were cut using a Leica UltraCut UC7. 60 nm sections were collected on
1250 formvar/carbon coated grids and contrast stained using 2% uranyl acetate and lead citrate. The
1251 sections were viewed in an FEI Tencai Biotwin TEM at 80 Kv. Images were taken using Morada
1252 CCD and iTEM (Olympus) software.

1253

1254 **Protein extractions**

1255 Mouse hearts were dissected and the blood was removed by flushing with PBS. The hearts were
1256 flash frozen in liquid nitrogen and homogenized on Mikro Dismembrator II. Cells were removed
1257 from the plates using accutase (for iPSC and M cells) or Trypsin-EDTA 0.25% (for CMs).
1258 Proteins were extracted using NE-PER Nuclear and Cytoplasmic extraction reagents (Thermo
1259 fisher scientific 78833) supplemented with proteinase inhibitors (Roche) according to

manufacturer instructions. After the fractions were acquired, they were combined. The proteins were analyzed by western blotting.

Histone extractions

Histones were extracted from the pellets from the protein extractions using the total histone extraction kit (Epigentek) according to manufacturer instructions. The extracted histones were analyzed by western blotting.

Western blots

The protein samples were evenly loaded onto 4-12% gradient Bis-Tris gels (Invitrogen) (UBE2B) or 3-8% Tris-Acetate gels (NuPAGE) (RNF20 and RNF40), transferred to PVDF membrane, blocked overnight in 5% dry milk in TBST, and incubated with primary antibodies overnight rocking at 4°C (**Supplemental Table 2**), followed by washes in TBST for 1 hour, incubated with corresponding HRP-conjugated secondary antibodies (Abcam ab6721, 1:20,000) for 1 hour at RT, washed in TBST for 4.5 hours. Images were acquired using film and scanned in to be quantified using imageJ⁶⁹. For a loading control for the wild-type mice over time (Fig. S1e), we used a Mini-PROTEAN TGX Stain-Free precast gel (Bio-Rad). For a loading control for the iPSCs, we used GAPDH (Fig. 4, S6). For a loading control for the *RNF20* mutant mice, we used alpha-tubulin (Fig. 4, S6).

The histone samples were evenly loaded onto 4-12% gradient Bis-Tris gels (Invitrogen), transferred to PVDF membrane, blocked for 1 hour in 5% dry milk in TBST, and incubated with primary antibodies overnight rocking at 4°C (**Supplemental Table 2**). The next day, the blots

1283 were washed in TBST three times for 15 minutes, incubated with corresponding HRP-conjugated
1284 secondary antibodies (Abcam ab6721, 1:20,000) for 1 hour at RT, washed in TBST 3 times for
1285 15 minutes. Images were acquired using film and scanned in to be quantified using imageJ⁶⁹.
1286 Expression was normalized to levels of H2B.

1287

1288 **ChIP-seq**

1289 Samples were collected in triplicate, where each replicate came from a plate that started
1290 differentiation at a different time. Samples were harvested by adding 2 mL of 1% formaldehyde
1291 to the media for 10 minutes. The reaction was quenched by adding 664.0625 mM of glycine for
1292 5 minutes. Two washes with ice cold PBS were done before scraping the cells of the plate in
1293 PBS using a cell scraper. The tube was spun down at 2000 rpm for 5 minutes and flash frozen in
1294 liquid nitrogen. The frozen samples were resuspended in 1 mL lysis buffer (10 mM Tris, 2 mM
1295 EDTA, and 0.1% SDS) for 30 minutes on ice and 1-6 wells of a 6-well plate (depending on the
1296 stage and how many cells were in each well) from one batch of cells were combined in the same
1297 tube. The DNA quantity was estimated by using a Qiagen PCR purification kit and the volumes
1298 were normalized to 6.25 ng/ μ L. The cells continued to lyse overnight. The samples were
1299 sonicated on a Bioruptor Pico (23 cycles of 30 seconds on and 30 seconds off) in 0.65 mL tubes
1300 with 120 μ L of sample in each tube. The DNA quantity was adjusted to 1 μ g per sample, diluted
1301 2 parts dilution buffer to 1 part DNA with a dilution buffer, and incubated with 4 μ L of H2Bub1
1302 antibody or IgG antibody overnight. It was then incubated with 50 μ L of protein G Dynabeads
1303 (Invitrogen) for 4 hours at 4°C while rocking. The beads were washed twice with low salt buffer,
1304 twice with high salt buffer, twice with LiCl buffer, and twice with TE buffer (changing tubes
1305 after the first TE wash). Each wash was three minutes long. The immunoprecipitated chromatin

was eluted from the beads in 85 μ L of elution buffer two times for 15 minutes at 70°C. The immunoprecipitated chromatin was then treated with RNase, underwent reverse cross-linking, and treated with proteinase K at 65°C overnight. The DNA was purified using a Qiagen PCR purification kit. qPCR was performed on these samples to verify that the immunoprecipitation worked using Quantabio PerfeCta syberGreen mix. The samples were run on either a 7900HT Fast Real-Time PCR Detection System (Applied Biosystems) or a CFX96 Touch Real-Time (BioRad) in triplicate. The results were analyzed by calculating percent input. The samples that passed quality control were sequenced at Yale Center for Genome Analysis using NovaSeq.

Buffers

Dilution Buffer: 16.7 mM Tris pH = 8, 167 mM NaCl, 1.2 mM EDTA, 0.002% SDS, 1.1% Triton, 1X proteinase inhibitors (Roche), and 5 mM Na butyrate)

Low Salt Buffer: 20 mM Tris pH = 8, 0.1% SDS, 1% Triton, 2 mM EDTA, 150 mM NaCl

High Salt Buffer: 20 mM Tris pH = 8.0, 0.1% SDS, 1% Triton, 2 mM EDTA, 500 mM NaCl

LiCl Buffer: 10 mM Tris pH = 8, 250 mM LiCl, 1 mM EDTA, 1% NP40, 1% Na-deoxycholate

TE Buffer: 10 mM Tris pH = 8, 1 mM EDTA

Elution Buffer: 50 mM Tris pH = 8, 1 mM EDTA, 1% SDS

ChIP-seq analysis

The ChIP-seq reads were trimmed using the FASTX-Toolkit to remove poor quality reads and were aligned to the human reference genome (hg38) using the BWA Mem algorithm (version 0.7.17). To call peaks, Model-based Analysis of ChIP-seq (MACS) (version 2.1.1.20160309) was used, where the input was used as the control, and reads were normalized using fold enrichment⁷⁰. Since H2Bub1 peaks are atypical, the BAMPE option, the no model option, and the broad option (with a cutoff of 0.2) were used. The presence of H2Bub1 near the TSS (-2 Kb, 10) as computed using “computeMatrix” in deepTools (version 2.5.0.1) and the genes were divided into four clusters using kmeans clustering based on the iPSC peaks (Fig. 2a, 2b) or the wild-type peaks (Fig. 5c, S5c, S8c)⁷¹. Four clusters were selected unbiasedly by implementing the elbow method in Python (version 2.7.13). The plots that were generated in deepTools represent the average of the three replicates. To determine the differentially bound sites between samples the “diffbind” package in R (version 3. .1) as used with the following parameters: maximum q value of 0.05 and minimum absolute fold change of 1.5^{72,73}. These differentially bound sites were matched to the nearest gene using the “ChIPseeker” package in R (UCSC hg38)^{73,74}. Importantly, a gene was only considered differentially bound (between the mutants and the wild-type cells) if there was a differentially bound region in the gene in both mutants. The heat maps were made by selecting the most differentially bound regions in each gene ontology () term using the “pheatmap” package in R^{73,75}. The Venn Diagrams were made using the R package VennDiagram^{73,76}. GO enrichment analysis was done in DAVID (version 6.8)^{77,78}. The gene traces were visualized in the Integrative Genomics Viewer (version 2.4.5)⁷⁹.

1352 **RNA-seq**

1353 Samples were collected in triplicate, where each replicate came from a plate that started
1354 differentiation at a different time. Each replicate consisted of two wells of a 6-well plate that
1355 were differentiated at the same time. Total RNA was extracted using a Qiagen RNeasy mini kit
1356 with the RNase-Free DNase Set. The mRNA-seq libraries were prepared using a KAPA mRNA
1357 HyperPrep Kit (cat no. 08098123702). Samples were sequenced at Yale Center for Genome
1358 Analysis using NovaSeq.

1359

1360 **RNA-seq analysis**

1361 The RNA-seq reads were trimmed using the FASTX-Toolkit to remove poor quality reads and
1362 were aligned to the human reference genome (hg38) using Kallisto (version 0.45.0) in R (version
1363 3.6.1)^{73,80}. Differential expression analysis was done using the Wald test in Sleuth (version
1364 0.30.0) in R with the following parameters: maximum q value of 0.05 and minimum absolute
1365 fold change of 1.5^{73,81}. BiomaRt (version 3.13) was used to match the transcripts with
1366 genes^{73,82,83}. As with ChIP-seq, a gene was only considered to be differentially expressed
1367 (between the mutants and the wild-type cells) if there was a differentially expressed transcript for
1368 the gene in both mutants. The heat maps were made by selecting the most differentially
1369 expressed regions in each term using the “pheatmap” package in R^{73,75}. GO enrichment
1370 analysis was done in DAVID (version 6.8)^{77,78}. The gene traces were visualized in the Integrative
1371 Genomics Viewer (version 2.4.5)⁷⁹.

1372

1373

1374

1375 **Analysis of compartment data**

1376 H2Bub1 peaks that overlapped by greater than or equal to 20 positions to a compartment
1377 interval, based on previously collected compartment data, were considered to be part of that
1378 interval³⁸. Intervals that had a positive value were in the “ ” compartment and intervals that had
1379 a negative value were in the “ ” compartment. The three ChIP-seq replicates were compared to
1380 the compartment data separately and then mean and SEM were calculated.

1381

1382 To calculate expected values, 1000 bootstrap replicates were created for each stage of CM
1383 differentiation. A bootstrap replicate was created by sampling the compartment file with
1384 replacement until the bootstrap replicate file had the same number of rows as the H2Bub1 peak
1385 file. By doing this, we generated a “random” collection of intervals from the “ ” and “ ”
1386 compartment that was of the same length as the H2Bub1 peak files. The mean and SEM were
1387 calculated on these files to get the expected values.

1388

1389 To calculate p-values between observed and expected, the following equation was used for each
1390 replicate: $p = \frac{\sum_1^n E_n > O}{n}$, where E is the expected percentage, O is the observed percentage, and n
1391 is the bootstrap replicate number. A sample was considered significant if every replicate had a p-
1392 value less than 0.05.

1393

1394 To calculate p-values between observed values of different stages, the following equation was
1395 used to calculate a z-score between these two percentages: $z = \frac{p_1 - p_2}{SE}$, where p₁ and p₂ are the
1396 percents and SE is the standard error. The z distribution was used to calculate a p-value from the
1397 z-score. All of this analysis was done in R (version 3.6.1)⁷³.

Analysis of histone marks

H3K27me3 and H3K4me3 peaks were downloaded from the Encode Consortium and generated by the Bernstein lab^{39,40}. Previously collected data on other histone marks from iPSC and CM stages, as well as the H2Bub1 data from the iPSC and CM stages, were matched to the nearest gene using the “ChIPseeker” package in R (UCSC hg38)^{39,40,73,74}. See the table below for which ENCODE files were used. For each replicate of every mark at each stage, the presence or absence of a peak in a gene was recorded in a data frame (1 indicated presence, and 0 indicated absence). A 2X2 contingency table was created to record how many genes contained both marks, how many genes had one mark, and how many genes had neither mark.

To calculate expected values, fixed row and column sums were used. For example, in a 2X2 contingency table, the expected value of position (2,2) is $E_{(2,2)} \frac{mk}{total}$, where m is the column sum and k is the row sum⁸⁴. The graph was made by plotting the mean ratios of the observed values to the expected values. Chi Square test was done to calculate p-values between observed values and expected values. P-values comparing observed values between stages were calculated using an unpaired 2-tailed, heteroscedastic t-test. All analyses were done in R (version 3.6.1)⁷³.

Experiment	Bedfile	Target	Tissue
ENCSR864LRY	ENCFF289NJD	H3K27me3	cardiac muscle originated from RUES2
ENCSR000ALU	ENCFF296RYM	H3K27me3	Human h1-hESC
ENCSR652QNW	ENCFF905NTT	H3K4me3	cardiac muscle originated from RUES2
ENCSR000AMG	ENCFF668YOE	H3K4me3	Human h1-hESC

1418 Analysis of patterns

1419 We wanted to investigate the drop off in H2Bub1 on gene bodies between the CMes and CP
1420 stages. Previously, a program was developed that used matrix factorization to investigate patterns
1421 in ChIP-seq and RNA-seq data⁵². Briefly, matrices are created where the rows are genes and the
1422 columns are positions along the gene. The matrices (one for each condition, in this case CMes
1423 and CP) get factored into two matrices, see diagram below. These two matrices when multiplied
1424 together yield the original matrix. The numbers get rearranged in each of these two submatrices
1425 such that genes with similar signals get grouped together.

1426

$$1427 \begin{bmatrix} g_1, po_1 & \cdots & g_1, po_n \\ \vdots & \ddots & \vdots \\ g_n, po_1 & \cdots & g_n, po_n \end{bmatrix} = \begin{bmatrix} g_1, pa_1 & \cdots & g_1, pa_n \\ \vdots & \ddots & \vdots \\ g_n, pa_1 & \cdots & g_n, pa_n \end{bmatrix} * \begin{bmatrix} pa_1, po_1 & \cdots & pa_1, po_n \\ \vdots & \ddots & \vdots \\ pa_n, po_1 & \cdots & pa_n, po_n \end{bmatrix}, \text{ where } g \text{ is}$$

1428 a gene, po is a position along the gene, and pa is a pattern.

1429

1430 It is unclear if the amount of H2Bub1 at a particular location is important, or whether the
1431 presence of H2Bub1 at a particular location is important. Therefore, we created two versions of
1432 these matrices.

1433

1434 Version one considers both distribution and amount. To create this version, we used
1435 “computeMatrix” in deepTools (version 2.5.0.1)⁷¹. We used the scale regions option to be able to
1436 analyze the entire gene. We limited our search from 2 Kb upstream of the TSS to 2 Kb
1437 downstream of the TES. We used 5000 for the region body length, which gave us 900 bins. We
1438 combined the three replicates together by averaging in R (version 3.6.1)⁷³.

1439

1440 Version two considers only distribution. To create this version, we went line by line in the
1441 H2Bub1 peak files using R (version 3.6.1) code⁷³. For each peak, we used interval searching to
1442 search through the ENSEMBL genes (this file was sorted by chromosome and the genes were in
1443 order based on position on the chromosome) to find all of the ENSEMBL genes each peak
1444 overlapped with. We divided each ENSEMBL gene into 100 bins of equal size. A peak was
1445 considered to be in a bin if it overlapped with more than a quarter of the bin. This created a 3d
1446 matrix, where the rows were genes, the columns were positions along the gene, and the third
1447 dimension was the replicates. To combine the replicates, a peak was considered present if it was
1448 present in a given bin of a given gene in at least two replicates.

1449

1450 We ran these four matrices (CMes version 1, CMes version 2, CP version 1, and CP version 2)
1451 through the previously published pipeline. After some tweaking of the K values to get a K value
1452 that is more representative of the global minimum, we decided to make K go from 2-8 for
1453 version 1 and K go from 2-7 for version 2. K represents the number of patterns. The K values
1454 were manually checked. In both cases, no common patterns emerged. The K value for CMes was
1455 seven and for CP was five in version 1. The K value for CMes was six and for CP was four in
1456 version 2. This program produced the graphs in Fig. S4a. To create Fig. S4b, the genes in each
1457 pattern from CMes in each version were compared to the differentially occupied genes identified
1458 using diffbind.

1459

1460 To create Fig. S4c, the genes in each pattern were combined with the RNA-seq data. The
1461 transcript with the highest expression level in the CMes stage for a given gene was used for both
1462 the CMes stage and the CP stage. Expression levels for each gene in each pattern at both stages

1463 were recorded in a matrix in R (version 3.6.1)⁷³. The box and whisker plots were plotted using
1464 ggplot2^{73,85}.

1465

1466 **Mouse calcium qRT-PCR**

1467 e11.5 mouse hearts were dissected and flash frozen in liquid nitrogen separately. After
1468 genotyping, 3-10 hearts were pooled together to form each sample. RNA was extracted with the
1469 Qiagen RNeasy Plus mini kit. cDNA was synthesized with Quantabio qScript cDNA SuperMix.
1470 qPCR was done using Quantabio PerfeCta syberGreen mix on a CFX96 Touch Real-Time
1471 (BioRad) in biological duplicates and technical triplicates. See Supplemental Table 1 for primers.
1472 Expression was normalized to the ribosomal protein *18s*.

1473

1474 **Movies**

1475 Movies of the CM stage cells were produced using bright-field imaging on the Zeiss Observer
1476 Z1. We collected 50 frames, 1 per second.

1477

1478 **Metagene analysis**

1479 We first assembled a list of regions. We decided to focus on calcium signaling genes and
1480 sarcomeric genes, since those are the genes that are differentially expressed between the ChIP-
1481 seq and RNA-seq data. We combined the sets of genes that were selectively maintained and
1482 differentially expressed into two bed files (one for calcium signaling genes and one for
1483 sarcomeric genes). We used the R package metagene2 (version 1.8.0) to create the plots with the
1484 following parameters: normalize with RPM, alpha = 1, and paired_end is true^{73,86}.

1485

1486 To generate “random” set of genes, we fit the lengths of the genes in the calcium signaling bed
1487 file and the sarcomeric gene bed file to the poisson function in R (version 3.6.1)⁷³. We then
1488 generated random numbers (the same number as there are lines in each bed file) from that
1489 distribution. For each random number, we found all of the down regulated genes in *UBE2B*^{-/-}
1490 that are within 10% of that number. We then kept picking a gene at random until one was
1491 downregulated in both mutants. We repeated the procedure for upregulated and non-regulated
1492 genes. We created 10 sets of each (upregulated calcium signaling, downregulated calcium
1493 signaling, non-regulated calcium signaling, upregulated sarcomeric genes, downregulated
1494 sarcomeric genes, and non-regulated sarcomeric genes). To verify that there was no
1495 accumulation near the center, these 60 graphs were manually inspected.

1496

1497 **Transcriptional efficiency**

1498 To calculate transcriptional efficiency, we used the R package CoverageView (version
1499 1.30.0)^{73,87}. We modified the source code of draw.heatmap to produce a pdf file to make it
1500 compatible with the machine it was being run on. We used 100 windows, normalized with RPM,
1501 and presented the graph as a log2ratio. We produced one graph per replicate per mutant. Since all
1502 of the replicates look similar and both mutants look similar, we show one representative image.
1503 We used the same set of “random” genes generated for the metagene analysis. We manually
1504 inspected the 360 resulting graphs (ten replicates of “random” genes for three classes of genes
1505 (upregulated, downregulated, and non-regulated), for two bed files (calcium signaling genes and
1506 sarcomeric genes) for three replicates of two mutant cell lines). The gene traces were visualized
1507 in the Integrative Genomics Viewer (version 2.4.5)⁷⁹.

1508

mESC and MEF analysis

We downloaded the raw data from previously published work and put it through our ChIP-seq and RNA-seq pipelines described in the ChIP-seq and RNA-seq analysis sections³². To determine the tissue-specific genes for the MEFs, we used the Wald test in Sleuth (version 0.30.0) (maximum q value of 0.05 and minimum absolute fold change of 1.5) to compare the wild-type MEFs to the wild-type mESCs^{73,81}. BiomaRt was used to match the transcripts with genes^{73,82,83}. GO enrichment analysis was done using DAVID (version 6.8) on the genes upregulated in the MEFs compared to the mESCs^{77,78}. The GO terms were related to ions, adhesion, and/or ECM. Thus, we created metagenes for genes in those three categories following the pipeline described in the metagene section. Since this group did not knockout a component of the RNF20-complex, we did not think it was necessary to create “random” genes representing the upregulated, downregulated, or non-regulated genes. Therefore, we created 20 “random” gene sets for ions, 20 “random” gene sets for adhesion, and 20 “random” gene sets for ECM.

Statistics and reproducibility

Graphs and statistics were either done in R (version 3.6.1) or Prism (version 9.1.0)⁷³. All experiments were done in replicate, with the number clearly indicated on the figure or in the figure legend. All statistical tests are defined in the figure legend and any non-traditional tests are described in detail in the appropriate section of the online methods. Multiple comparison adjustments were done using Bonferroni method. Biological replicates are samples analyzed from distinct samples. To account for off-target effects due to CRISPR, we created two cell lines for each mutant. For mouse experiments, since multiple mice were pooled together into a

1531 replicate, both sexes were mixed together in each sample. Additionally, littermate controls were
1532 used. The iPSC experiments are all done in a male cell line.

1533

1534 **Data Availability**

1535 Requests for mice, induced pluripotent stem cell lines, or reagents will be granted by the
1536 corresponding author. The ChIP-seq and RNA-seq data will be deposited in GEO and we will
1537 list the accession numbers here. The other ChIP-seq and RNA-seq data sets that we used are
1538 available from the following resources. The A and B compartment data are available under GEO
1539 accession GSE106690. The histone mark data are available on ENCODE; see online methods for
1540 ENCODE accessions. The MEF data are available under GEO accession GSE153587.

1541

1542 **Code Availability**

1543 All software used in this study is publicly available. The code for figures and analysis is
1544 available upon request.

References

32. Wang, F. *et al.* Histone H2Bub1 deubiquitylation is essential for mouse development, but does not regulate global RNA polymerase II transcription. *Cell Death Differ* (2021).
37. Lian, X. *et al.* Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/beta-catenin signaling under fully defined conditions. *Nat Protoc* **8**, 162-75 (2013).
38. Bertero, A. *et al.* Dynamics of genome reorganization during human cardiogenesis reveal an RBM20-dependent splicing factory. *Nat Commun* **10**, 1538 (2019).
39. Inoue, F. *et al.* A systematic comparison reveals substantial differences in chromosomal versus episomal encoding of enhancer activity. *Genome Res* **27**, 38-52 (2017).
40. Kazachenka, A. *et al.* Identification, Characterization, and Heritability of Murine Metastable Epialleles: Implications for Non-genetic Inheritance. *Cell* **175**, 1259-1271 e13 (2018).
52. Zhang, L. & Zhang, S. Learning common and specific patterns from data of multiple interrelated biological scenarios with matrix factorization. *Nucleic Acids Res* **47**, 6606-6617 (2019).
68. Chen, Y. *et al.* Engineering Human Stem Cell Lines with Inducible Gene Knockout using CRISPR/Cas9. *Cell Stem Cell* **17**, 233-44 (2015).
69. Schneider, C.A., Rasband, W.S. & Eliceiri, K.W. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* **9**, 671-5 (2012).
70. Zhang, Y. *et al.* Model-based analysis of ChIP-Seq (MACS). *Genome Biol* **9**, R137 (2008).
71. Ramirez, F. *et al.* deepTools2: a next generation web server for deep-sequencing data analysis. *Nucleic Acids Res* **44**, W160-5 (2016).
72. Stark, R. & Brown, G. DiffBind: differential binding analysis of ChIP-Seq peak data. Bioconductor <http://bioconductor.org/packages/release/bioc/vignettes/DiffBind/inst/doc/DiffBind.pdf>. (2011).
73. R Core Team. R: A language and environment for statistical computing. (R Foundation for Statistical Computing, Vienna, Austria, 2019).
74. Yu, G., Wang, L.G. & He, Q.Y. ChIPseeker: an R/Bioconductor package for ChIP peak annotation, comparison and visualization. *Bioinformatics* **31**, 2382-3 (2015).
75. Kolde, R. pheatmap: Pretty Heatmaps. R package version 1.0.8. <https://CRAN.R-project.org/package=pheatmap>. (2015).
76. Chen, H. & Boutros, P.C. VennDiagram: a package for the generation of highly-customizable Venn and Euler diagrams in R. *BMC Bioinformatics* **12**, 35 (2011).
77. Huang da, W., Sherman, B.T. & Lempicki, R.A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* **4**, 44-57 (2009).
78. Huang da, W., Sherman, B.T. & Lempicki, R.A. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* **37**, 1-13 (2009).
79. Thorvaldsdottir, H., Robinson, J.T. & Mesirov, J.P. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Brief Bioinform* **14**, 178-92 (2013).

1588 80. Bray, N.L., Pimentel, H., Melsted, P. & Pachter, L. Near-optimal probabilistic RNA-seq
1589 quantification. *Nat Biotechnol* **34**, 525-7 (2016).

1590 81. Pimentel, H., Bray, N.L., Puente, S., Melsted, P. & Pachter, L. Differential analysis of RNA-
1591 seq incorporating quantification uncertainty. *Nat Methods* **14**, 687-690 (2017).

1592 82. Durinck, S. *et al.* BioMart and Bioconductor: a powerful link between biological
1593 databases and microarray data analysis. *Bioinformatics* **21**, 3439-40 (2005).

1594 83. Durinck, S., Spellman, P.T., Birney, E. & Huber, W. Mapping identifiers for the integration
1595 of genomic datasets with the R/Bioconductor package biomaRt. *Nat Protoc* **4**, 1184-91
1596 (2009).

1597 84. Fu, A.Q. & Adryan, B. Scoring overlapping and adjacent signals from genome-wide ChIP
1598 and DamID assays. *Mol Biosyst* **5**, 1429-38 (2009).

1599 85. Wickham, H. ggplot2: Elegant Graphics for Data Analysis. (Springer-Verlag New York. ,
1600 2016).

1601 86. Fournier, E., Joly Beauparlant, C., Lippens, C. & Droit, A. metagene2: A package to
1602 produce metagene plots. R package version 1.8.0. (2021).

1603 87. Lowry, E. CoverageView: Coverage visualization package for R. R package version 1.30.0.
1604 (2021).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementalData6UBE2BRNAseq.xlsx](#)
- [SupplementalData4RNF20RNAseq.xlsx](#)
- [Movie4RNF202NotBeating.mov](#)
- [Movie3RNF201NotBeating.mov](#)
- [SupplementalData2WildTypeRNAseq.xlsx](#)
- [SupplementalData5UBE2BChIPseq.xlsx](#)
- [SupplementalData3RNF20ChIPseq.xlsx](#)
- [Movie2RNF201Beating.mov](#)
- [Movie6UBE2B1NotBeating.mov](#)
- [Movie8UBE2B2NotBeating.mov](#)
- [SupplementalData1WildTypeChIPseq.xlsx](#)
- [Movie1wildtype.mov](#)
- [Movie5UBE2B1Beating.mov](#)
- [Movie7UBE2B2Beating.mov](#)