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Maintenance of adult cardiac function requires the chromatin factor Asxl2

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Abstract

During development and differentiation, cell type-specific chromatin configurations are set up to facilitate cell type-specific gene expression. Defects in the establishment or the maintenance of the correct chromatin configuration have been associated with diseases ranging from leukemia to muscular dystrophy. The heart expresses many chromatin factors, and we are only beginning to understand their roles in heart development and function. We have previously shown that the chromatin regulator Asxl2 is highly expressed in the murine heart both during development and adulthood. In the absence of Asxl2, there is a significant reduction in trimethylation of histone H3 lysine 27 (H3K27), a histone mark associated with lineage-specific silencing of developmental genes. Here we present evidence that Asxl2 is required for the long-term maintenance of ventricular function and for the maintenance of normal cardiac gene expression. *Asxl2*$^{-/-}$ hearts displayed progressive deterioration of ventricular function. By 10 months of age, there was ~37% reduction in fractional shortening in *Asxl2*$^{-/-}$ hearts compared to wild-type. Analysis of the expression of myofibril proteins suggests that *Asxl2* is required for the repression of βMHC. *Asxl2*$^{-/-}$ hearts did not exhibit hypertrophy, suggesting that the de-repression of β-MHC was not the result of hypertrophic response. Instead, Asxl2 and the histone methyltansferase Ezh2 co-localize to β-MHC promoter, suggesting that Asxl2 directly represses β-MHC. Interrogation of the CardioGenomics database revealed that *ASXL2* is down-regulated in the hearts of patients with ischemic or idiopathic dilated cardiomyopathy. We propose that chromatin factors like Asxl2 function in the adult heart to regulate cell type- and stage-specific patterns of gene expression, and

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DISCLOSURES

None.

the disruption of such regulation may be involved in the etiology and/or development of certain forms of human heart disease.

Keywords

Chromatin factor; ventricular dysfunction; β-MHC de-repression; histone methylation; human heart disease etiology

INTRODUCTION

Transcriptional regulation plays critical roles in heart development and function [1–6]. During embryonic development, the process of heart morphogenesis requires precise regulation of gene expression. Aberrations in the temporal or spatial pattern of gene expression lie at the root of multiple forms of congenital heart defects (CHD). Postnatally, gene expression is fine-tuned to meet the contractile need of adult life. There is little cardiomyocyte turnover in the adult heart, and the appropriate gene expression pattern has to be maintained for a life time. Mutations in a number of transcription factors or changes in the dosage of transcription factors have been shown to cause cardiac dysfunction in humans or in animal models, highlighting the importance of transcriptional regulation in the adult heart [7–11].

In recent years, chromatin has emerged as an important layer of transcriptional regulation. Many chromatin-associated proteins have been identified, and studies have shed light on how these chromatin factors can modify chromatin configuration to either facilitate or inhibit transcription. Accumulating evidence suggests that a substantial amount of transcriptional regulation in the heart takes place at the chromatin level [13–21]. However, much remains to be learned about which chromatin factors are involved, which genes they regulate, what functional mechanisms are used and whether/how deregulation contributes to heart diseases.

Polycomb Group (PcG) and Trithorax Group (TrxG) proteins are two highly conserved protein families that regulate transcription by modifying chromatin structure [22–25]. PcG proteins form several complexes to create repressive chromatin structure and maintain long-term silencing of target genes. For example, Polycomb Repressive Complex 2 (PRC2) generates trimethylated histone H3 lysine 27 (H3K27me3), a histone mark of silent chromatin. Polycomb Repressive Complex 1 (PRC1) has chromatin compaction activity. TrxG proteins also form multiple complexes but create active chromatin structure and antagonize PcG-mediated repression. Components of the PcG/TrxG system are expressed in the heart both during development and in the adult. Several pieces of evidence have implicated the system in transcriptional regulation in both embryonic and postnatal hearts. For example, studies of mice mutant for *Rae28* suggest that proper heart morphogenesis requires PcG activity. *Rae28* mutant mice display severe defects in an early and important step of cardiac morphogenesis, cardiac looping, which takes place between E8.5 and E9.5 [17, 18]. Postnatal over-expression of *Rae28* caused dilated cardiomyopathy, cardiomyocyte apoptosis, abnormal myofibrils, and severe heart failure [19]. The TrxG protein Brg1 promotes cardiomyocyte proliferation and regulates the activity of multiple cardiac transcription factors in a dosage-dependent manner during heart development [20, 21]. In adult cardiomyocytes, Brg1 is required for stress-induced hypertrophy and the pathological alpha-MHC to beta-MHC shift [20].

We have previously generated a mutant mouse model for the chromatin factor Asxl2. We showed that Asxl2 is an enhancer of PcG activity and that Asxl2 deficiency has a significant

impact on the level of bulk H3K27me3 [26]. In addition, two Asxl2 homologs have been shown to form a complex with the histone deubiquitinase Calypso/BAP1 and promote deubiquitination of histone H2A [27]. *Asxl2* is highly expressed in the heart throughout development and during adult life. To better understand the role of *Asxl2* in the heart, we carried out a longitudinal study of $Asxl2^{-/-}$ mice. Our data indicate that Asxl2 is required for the long-term maintenance of ventricular function and for repression of *β-MHC*. Asxl2 is likely a direct regulator of *β-MHC* and this regulation may involve the PcG protein Ezh2. Finally, *ASXL2* is down-regulated in human patients with ischemic or idiopathic dilated cardiomyopathy.

MATERIALS AND METHODS

Animal breeding

All mice used in this study were in C57BL/6J x 129Sv F1 background because $Asxl2^{-/-}$ animals in either C57BL/6J or 129Sv inbred background die perinatally. $Asxl2^{+/-}$ females in 129Sv inbred background were mated to $Asxl2^{+/-}$ males in C57BL/6J inbred background to produce $Asxl2^{-/-}$ animals and wild-type littermates. The genetic compositions of the experimental and control animals were identical except at the *Asxl2* locus.

Echocardiography

Transthoracic echocardiography was performed while under isoflurane anesthesia and positive pressure ventilation. Transthoracic two-dimensional targeted M-mode and pulsed-wave Doppler echocardiography was performed with a 30-MHz mechanical transducer attached to a VisualSonics Vevo 770 system (Visual Sonics, Toronto, ON, Canada).

Hemodynamic measurements

Hemodynamic measurements were performed on 5-month old wild-type and $Asxl2^{-/-}$ mice. Mice were anesthetized with isoflurane (1.5%) and injected with etomidate (10 mg/kg body weight; I.P.) for intubation. Anesthesia was maintained at 1% isoflurane and mice were ventilated with a Harvard Respirator at a rate of 140 breaths per minute and a 250 μm volume. A medial laparotomy exposed the diaphragm and a Millar Pressure/Volume transducer (SPR-839) was inserted into the left ventricle through an apical puncture. Steady state measurements of pressure/volume loops were recorded and the inferior vena cava was occluded to derive load-independent measurements of the end-systolic pressure/volume relation.

SDS-PAGE gel electrophoresis

Myofibril proteins were prepared and separated on SDS-PAGE as previously described (Arteaga 2005). The gels were subjected to either Coomassie staining to visualize all proteins or to Pro-Q Diamond staining (Molecular Probes, Eugene, OR) to visualize phosphorylated proteins. Alternatively, separated proteins were transferred to PVDF membrane and subjected to Western blot analysis.

For high-resolution SDS-PAGE, samples were loaded on 6% SDS-PAGE gels, run for 30 h at 4°C, and subjected to silver staining. Proportions of α- and β-MHC were determined using densitometry.

Blood pressure measurement

Blood pressures of male mice ranging from 1-month to 10-months of age were measured in unanesthetized mice using an NIBP-8 tail-cuff blood pressure monitor (Columbus Instruments, Columbus, Ohio). Animals were acclimated to the restrainer and the warming

compartment for 30 min./day for at least 3 days. On the day of the experiment, animals were acclimated in the apparatus for 20 minutes before measurements were taken. The sensor cuff pressure was set at 45 mmHg and the occlusion cuff pressure was 200 mmHg. Each data point (for one animal at a specific age) represents the average of 10 or more sequential measurements, spaced at a minimum of one minute intervals.

Real-time RT-PCR

Real-time RT-PCRs were performed on an ABI Prism 7900HT sequence detection system (Applied Biosystems) using the SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen). The expression level of each gene analyzed was normalized against that of 18S rRNA or *β-Actin* in the same sample. For each gene, two wild-type and two mutant animals were analyzed. Detailed information on primer sequences and PCR conditions is given in the Supplementary Material.

Chromatin immunoprecipitation

Nuclei were collected from formaldehyde-fixed, homogenized adult heart tissues. Chromatin was sheared by sonication and immunoprecipitated with KC17 anti-Asxl2 antibody, anti-Ezh2 antibody (AC22, Millipore) or rabbit IgG (Invitrogen). ChIP-ed DNA was analyzed by PCR using primers specific for conserved regions in the β-MHC promoter (b1–b5). Detailed conditions and primers sequences are provided in Supplementary Material.

Histology and Immunofluoresence

Paraffin embedded heart sections were made using standard protocols. H&E stainings were performed on 8um sections; wheat-germ agglutinin-fluorescein staining was performed on 5um sections.

Adult cardiomyocyte size measurement

Cardiomyocyte size was measured for two pairs of 6-month-old *Asxl2*$^{-/-}$ and wild-type hearts. Isolation of adult cardiomyocytes was performed as previously described [28]. Cardiomyocytes were plated in the presence of butanedione monoxime (BDM), a contraction inhibitor, and allowed to attach for 3 hours. Images of live cardiomyocytes were taken. The length, width and area of cells were measured with ImageJ. 124–262 individual cardiomyocytes were measured for each heart.

RESULTS

Asxl2 is required for the maintenance of ventricular function

Ventricular systolic function was measured by fractional shortening (FS) and ejection fraction (EF) at 2 months, 4 months and 10 months of age (Fig. 1). While FS and EF of *Asxl2*$^{-/-}$ hearts were comparable to that in wild-type littermates at 2 months, both parameters deteriorated over time. We observed reduced FS in *Asxl2*$^{-/-}$ mice at 4 months, and by 10 months FS was ~38% lower in *Asxl2*$^{-/-}$ mice compared to wild-type littermates (Fig. 1C). EF followed a similar trend and was ~30% lower in *Asxl2*$^{-/-}$ mice compared to wild-type littermates by 10 months (Fig. 1D). The progressive deterioration of contractility in *Asxl2*$^{-/-}$ mice suggests that *Asxl2* is required for long-term maintenance of ventricular function.

Ventricular dysfunction in older *Asxl2*$^{-/-}$ mice was confirmed by hemodynamic measurements. At 5 months, two *Asxl2*$^{-/-}$ mice exhibited decreased left ventricular pressure (61 and 81 mmHg) and maximum dP/dt (3368 and 4751 mmgHg/sec) compared to a wild-type littermate (108 mmHg and 6399 mmHg/sec). Furthermore, contractile performance as

measured by the end-systolic pressure volume relation (Ees) and time varying maximal elastance (Emax) were markedly lower in the two *Asxl2*$^{-/-}$ mice (Ees: 10.96 and 4.11 mmHg/μl; and Emax: 13.73 and 6.01 mmHg/μl) compared to the wild-type littermate (Ees: 14.8 mmHg/μl and Emax: 25.4 mmHg/μl). These data demonstrate that by 5 months of age, *Asxl2*$^{-/-}$ mice were already exhibiting depressed ventricular function, independent of loading conditions.

Asxl2$^{-/-}$ mice have low arterial blood pressure at older age

Because hypertension is often associated with systolic dysfunction [29], we asked if the *Asxl2*$^{-/-}$ mice had hypertension. Arterial blood pressure was measured in unanesthetized mice at six time points from 1 month to 10 months. Both systolic and diastolic arterial pressures were comparable between *Asxl2*$^{-/-}$ mice and wild-type littermates up through 6 months of age. Systolic blood pressure (SBP) in *Asxl2*$^{-/-}$ mice decreases sharply between 6 months and 8 months and was significantly lower than that of wild-type littermates at 8 months ($p = 0.0083$) and 10 months ($p = 0.0281$) (Fig. 3A). Diastolic blood pressure (DBP) was also lower in *Asxl2*$^{-/-}$ mice at 8 months and 10 months, and the difference with wild-type DBP reached statistical significance at 10 months ($p = 0.0385$) (Fig. 3B). Thus, *Asxl2*$^{-/-}$ mice are not hypertensive, and older *Asxl2*$^{-/-}$ mice exhibited a significant reduction in arterial blood pressure, which may be a secondary effect of systolic function impairment.

Asxl2$^{-/-}$ heart exhibits increased PKA signaling

To determine the molecular basis for ventricular dysfunction in *Asxl2*$^{-/-}$ mice, we compared the expression and phosphorylation of myofibril proteins in *Asxl2*$^{-/-}$ and wild-type heart. We observed an increase in the level of phosphorylated cardiac Troponin I (cTnI) in 4-month-old *Asxl2*$^{-/-}$ hearts on Pro-Q Diamond stained gels (data not shown). cTnI contains multiple phosphorylation sites. Of particular importance is phosphorylation at Ser23/Ser24 by PKA, which increases the kinetics of Ca^{2+} exchange with cTnC [30]. To examine the level of PKA-phosphorylated cTnI, we performed Western blot analysis using an antibody specific to cTnI phosphorylated at Ser23/Ser24. The levels of both total cTnI and PKA-phosphorylated cTnI in *Asxl2*$^{-/-}$ hearts were comparable to wild-type littermates at 2 months (Fig. 2A, B). However, the level of PKA-phosphorylated cTnI was significantly higher in *Asxl2*$^{-/-}$ hearts at 4 months (Fig. 2C, D). Western blot analysis of phospholamban (PLB), another PKA substrate, corroborated the cTnI result. PLB binds to the Ca^{2+} pump SERCA and inhibits SERCA function. Phosphorylation of PLB by PKA relieves this inhibition and activates SERCA Ca^{2+}-ATPase. At 2 months, we observed an increase in both total and phosphorylated PLB in *Asxl2*$^{-/-}$ heart (Fig. 2E–G). At 4 months, the level of total PLB is decreased in *Asxl2*$^{-/-}$ heart but the level of phosphorylated PLB is increased (Fig. 2H–J). Taken together, these results showed that PKA signaling was enhanced in *Asxl2*$^{-/-}$ heart. This enhancement was observed at a young age (2 months), when systolic function and blood pressure were both normal in *Asxl2*$^{-/-}$ mice. Therefore, it is likely a primary phenotype associated with the loss of *Asxl2*, instead of a secondary result of sympathetic activity stimulation via baroreflex.

Asxl2$^{-/-}$ hearts exhibit de-repression of β-MHC

A recent study showed that Brg1, a chromatin remodeling ATPase and the mammalian homolog of the *Drosophila* TrxG protein Brm, is required for re-expression of *β-MHC* during hypertrophic response [20]. The normal adult mouse heart only expresses α-MHC, which has a higher Ca^{2+} ATPase activity than β-MHC. Myosin fibers composed entirely of α-MHC have higher actin filament sliding velocity than those composed of a mixture of α- and β-MHC. Transgenic studies have shown that even a slight re-expression of β-MHC in the adult mouse heart can result in physiologically significant changes in cardiac

contractility [31]. Because PcG and TrxG proteins often act antagonistically on the same set of target genes, we reasoned that Asxl2, which is required for PcG activity, might have a role in the repression of β-MHC in the adult heart. We first used real-time RT-PCR to examine the transcript level of *β-MHC* in *Asxl2*$^{-/-}$ and wild-type hearts. One-month-old *Asxl2*$^{-/-}$ heart exhibited ~3 fold de-repression of *β-MHC* in comparison to wild-type (Fig 4A). The degree of de-repression increased with age, and the level of *β-MHC* transcript in 8-month-old *Asxl2*$^{-/-}$ heart was ~19 fold of that in the wild-type heart (Fig 4A). Real-time RT-PCR analysis of *α-MHC* showed that there was a ~40% decrease in *Asxl2*$^{-/-}$ heart at 1 month and ~50% increase at 8 months (Fig 4B).

We then examined the level of β-MHC protein with high-resolution SDS-PAGE. Expression of β-MHC protein could be consistently detected in protein extract from 4-month-old *Asxl2*$^{-/-}$ heart but never in the wild-type control heart (Fig. 4D). We could not detect β-MHC protein on SDS-PAGE in protein extract from 2-month-old *Asxl2*$^{-/-}$ heart despite real-time RT-PCR data suggesting that β-MHC was de-repressed as early as 1 month (Fig. 4C). While this may be due to different sensitivity of silver staining and real-time RT-PCR, both protein data and transcript data suggest that β-MHC de-repression in *Asxl2*$^{-/-}$ heart become more pronounced with age.

Asxl2 and the PcG protein Ezh2 colocalize to *β-MHC* promoter

We have previously shown that Asxl2 functions as an enhancer of PcG activity [26]. *Asxl2*$^{-/-}$ heart exhibited a significant reduction in the bulk level of H3K27me3, which is the product of PcG histone methyltransferase activity and a mark of silenced chromatin. To better understand the functional mechanism of Asxl2, we generated a polyclonal antibody against Asxl2, KC17. We made FLAG-tagged Asxl2 using a rabbit reticulocyte in vitro transcription/translation kit and performed Western blot analysis using either KC17 or the M2 monoclonal antibody against FLAG (Fig. 5A). KC17 and M2 recognized the same band in the FLAG-Asxl2 translation mixture, but not in the control luciferase translation mixture, confirming that KC17 recognizes Asxl2. Immunofluorescence of HEK293 cells transfected with a FLAG-tagged Asxl2 also confirmed that signals from KC17 and anti-FLAG antibody completely overlap (data not shown). Next, we performed chromatin immunoprecipitation (ChIP) assays with KC17 to examine the association between Asxl2 and the *β-MHC* promoter. It has been previously reported that there are 5 conserved regions within 5kb upstream of the *β-MHC* transcription start site [19]. The TrxG protein Brg1 is associated with 4 of these 5 regions. We found that Asxl2 was enriched at all 5 regions (Fig. 5B). Furthermore, the PcG protein Ezh2, which is the histone methyltransferase responsible for PcG's histone methylation activity, co-localized with Asxl2 at 4 out of 5 of these regions. These results suggest that Asxl2 plays a direct role in the repression of *β-MHC*, and this repression may involve Ezh2 histone methyltransferase activity.

Asxl2$^{-/-}$ hearts are not hypertrophic

De-repression of *β-MHC* is often observed during hypertrophy, when the heart responds to stress by increasing the size of cardiomyocytes. De-repression of *β-MHC* in *Asxl2*$^{-/-}$ heart could be the result of hypertrophic response – thus a secondary effect to ventricular dysfunction. Alternatively, it could be a primary effect of the loss of *Asxl2* and (at least part of) the cause of ventricular dysfunction. Indeed, transgenic expression of *β-MHC* can cause ventricular dysfunction without inducing hypertrophy [31]. To distinguish the two possibilities, we examined the histology of *Asxl2*$^{-/-}$ heart for signs of hypertrophy. *Asxl2*$^{-/-}$ heart were not hypertrophic at any stage examined (Fig. 6A, B). WGA staining of wild-type and *Asxl2*$^{-/-}$ heart section showed that cardiomyocyte diameter was comparable (Fig. 6C, D). To further evaluate the size of cardiomyocytes, cardiomyocytes were isolated from 6-month-old wild-type and *Asxl2*$^{-/-}$ hearts. Consistent with the WGA staining result, wild-

type and *Asxl2*$^{-/-}$ cardiomyocytes exhibited similar width (Fig. 6E). However, in comparison to wild-type, the average length of *Asxl2*$^{-/-}$ cardiomyocytes was 13% shorter, and the average area was 17% smaller (Fig. 6F, G). In general, *Asxl2*$^{-/-}$ hearts had a higher proportion of shorter cardiomyocytes than wild-type hearts (Supplementary Fig. S1). We conclude that *Asxl2*$^{-/-}$ hearts did not develop cellular hypertrophy.

The gene *Nppa*, which encodes the atrial natriuretic factor (ANP), is normally highly expressed in the atria but not in the ventricles. Re-expression of *Nppa* in the ventricles is observed during hypertrohic response and considered a molecular hallmark of hypertrophy. We examined the expression of *Nppa* in *Asxl2*$^{-/-}$ LV by real-time RT-PCR. Instead of observing *Nppa* de-repression, we observed ~67% down-regulation in *Asxl2*$^{-/-}$ LV at 1 month and ~52% down-regulation at 8 months (Fig. 6E). Therefore, *Asxl2*$^{-/-}$ heart did not develop hypertrophic response at the molecular level.

ASXL2 is down-regulated in the hearts of patients with ischemic or idiopathic dilated cardiomyopathy

The CardioGenomics project has analyzed global gene expression pattern in the hearts of healthy individuals and patients with ischemic or idiopathic dilated cardiomyopathy by microarray. Expression in the LV was compared between 11 organ donors whose heart could not be used in transplant, 15 patients with heart failure arising from idiopathic dilated cardiomyopathy and 11 patients with heart failure arising from ischemic cardiomyopathy. We examined the expression of *ASXL* genes in the publically available microarray dataset (http://cardiogenomics.med.harvard.edu/public-data). There are three probe sets designated as *ASXL2* on the HgU133 Plus 2.0 array but only two of them, 218658_at and 1555266_a_at, map to the *ASXL2* locus. The third probe set, 226251_at, maps to an intergenic region downstream of *ASXL2* 3′UTR. Although 218659_at consistently showed higher signal intensity than 1555266_a_at, the two probe sets displayed the same trend showing that *ASXL2* expression is significantly down-regulated in both idiopathic and ischemic cardiomyopathy patients (Fig. 7). This suggests that the reduction of *ASXL2* expression may play a role in the etiology of some types of human cardiomyopathy. We were not able to determine with certainty whether the expression of *ASXL1* changes in human cardiomyopathy based on the microarray data, because different probe sets (all of which map correctly to the *ASXL1* locus) gave different results (data not shown). *ASXL3* expression was not detected above noise level in the microarray dataset (data not shown).

DISCUSSIONS

The molecular basis for ventricular dysfunction in *Asxl2*$^{-/-}$ heart

Contractility of *Asxl2*$^{-/-}$ heart was comparable to that in wild-type at young age but deteriorated progressively over time. This suggests that Asxl2 has a specific role in the long-term maintenance of ventricular function. In parallel with the progressive decrease in contractility, we observed progressive de-repression of *β-MHC* in *Asxl2*$^{-/-}$ heart. The de-repression of *β-MHC* was not a secondary result of stress-induced hypertrophy, since *Asxl2*$^{-/-}$ heart did not develop hypertrophy. Given that the TrxG protein Brg1 has been shown to be directly required for *β-MHC* activation [20], we reasoned that Asxl2, a PcG protein, may be directly required for *β-MHC* repression. ChIP assays showed that Asxl2 is enriched at the *β-MHC* promoter, supporting a directly role for Asxl2 in *β-MHC* repression. Thus, *β-MHC* de-repression was likely a direct consequence of *Asxl2* deficiency. Interestingly, Brg1 functions not only as a *β-MHC* activator but also as an *α-MHC* repressor [20]. The role of Asxl2 in *α-MHC* expression remains to be addressed, but it is formally possible that Asxl2 normally stimulates *α-MHC* expression, in which case the loss of *Asxl2* could lead to both direct and compensatory de-repression of *β-MHC*.

It has been previously shown that even a slight change in myosin composition – 12% of total myosin being β-MHC - can result in significant reduction in contractility [31]. The protein level of β-MHC in *Asxl2*$^{-/-}$ heart is ~6% of total myosin at 4 months. While this is a small change in myosin composition, it could have a detectable impact on contractility. The proportion of β-MHC in total myosin is likely much higher by 10 months, given that *β-MHC* de-repression becomes more severe over age (Fig. 4A). Thus we hypothesize that ventricular dysfunction in *Asxl2*$^{-/-}$ heart could, at least partly, result from a failure to maintain *β-MHC* in a silenced state.

Interestingly, *Asxl2*$^{-/-}$ heart exhibited increased PKA-phosphorylation of cTnI and PLB, both of which should increase the kinetics of Ca^{2+} transient and enhance contraction. Thus, contractility in *Asxl2*$^{-/-}$ heart reflects the net result of a tug-of-war between abnormal myosin composition and enhanced PKA signaling. Additional, as-yet unidentified factors may also contribute to this tug-of-war. A proteomic approach would be well suited for the identification of such factors in the future.

The role of Asxl2 in cardiomyocyte hypertrophy

Systolic dysfunction usually induces cardiomyocyte hypertrophy as a compensatory mechanism. However, there is a disconnection between systolic function and hypertrophy in *Asxl2*$^{-/-}$ hearts. This is uncommon but not unprecedented. For example, *β-MHC* transgenic mice have been reported to exhibit systolic dysfunction without developing hypertrophy [31]. Furthermore, mutations in several genes - including the transcription factor *Gata4* and the chromatin factor *Brg1* - have been shown to significantly attenuate hypertrophic response induced by transverse aortic constriction (TAC), a powerful form of pressure overload that normally causes rapid development of hypertrophy [11, 20]. These reports suggest that while systolic function and cardiomyocyte growth are usually intricately connected, it is possible to uncouple them. An important task in future studies would be to characterize molecular pathways downstream of Asxl2, in particular pathways that regulate cellular growth. The ability to manipulate such pathways could facilitate the prevention of hypertrophy and its deleterious effects.

The role of chromatin factors in the long-term maintenance of cardiac gene expression and function

The adult mammalian heart has very limited regeneration capability. Cardiomyocytes need to maintain a cell type-appropriate gene expression pattern for a life time while constantly adjusting performance and metabolism in response to changes in physiological conditions, such as exercise, hormones, and pregnancy. Previous research has identified an array of transcription factors as important regulators of gene expression in the adult heart. Deficiency or mutation in these transcription factors lead to malfunction of cardiomyocytes and of the whole heart. For example, Mef2a, a typical transcription factor with both DNA binding and transactivating activities, is required for the maintenance of appropriate mitochondrial content and cyto-architectural integrity in the adult mouse heart [32]. In the past two decades, studies have revealed the importance of transcriptional regulation at the level of chromatin. Chromatin is a complex of genomic DNA and histones. Whether the chromatin at a specific gene locus is permissive or inhibitory for transcription is influenced by the density of nucleosomes, the affinity between nucleosomes and DNA, and the combination of histone modifications that serve as a platform to attract or repel regulatory factors. Once set up, chromatin configurations can be passed on to daughter cells, ensuring that the progenies of a cell faithfully adopt the mother cell's transcriptional profile. In terminally differentiated cells that no longer divide, locus-specific chromatin configurations help maintain the differentiated state of the cell by facilitating the expression of some genes and silencing the expression of others. Many chromatin factors have been reported. Among these, PcG

proteins are particularly recognized for their role in creating and maintaining gene silencing. TrxG proteins, the antagonists of PcG proteins, can relieve PcG-mediated silencing.

It has recently been shown that the TrxG protein Brg1 is required for MHC isoform switching during hypertrophic response in the adult mouse heart [20]. Brg1 binds to 4 of the 5 conserved regions in the *β-MHC* promoter and can activate *β-MHC* reporters in an HDAC-independent manner. If a TrxG protein is required for *β-MHC* activation, do PcG proteins play a role in *β-MHC* repression? Our results suggest the answer is yes. Asxl2 is a chromatin factor and a regulator of histone methylation [26] and histone ubiquitylation [*H. Lai, unpublished results*]. *Asxl2*$^{-/-}$ hearts had decreased levels of bulk H3K27me3, a mark for silenced chromatin, suggesting that Asxl2 normally promotes PcG-mediated gene repression. Here we report that *β-MHC* is progressively de-repressed in *Asxl2*$^{-/-}$ hearts; furthermore, Asxl2 and the PcG histone methyltransferase Ezh2 co-localize to 4 out of 5 conserved regions in the *β-MHC* promoter. Thus, the expression of *β-MHC* is likely controlled by opposing actions of PcG and trxG proteins. In this model, PcG, with the help of Asxl2, functions to keep the *β-MHC* locus in a silenced chromatin configuration. In the absence of *Asxl2*, PcG activity is reduced, resulting in progressive deterioration of the silenced chromatin configuration and thereby progressive *β-MHC* de-repression. It is conceivable that there are more genes that require Asxl2/PcG for repression in the adult heart. The identification and study of these genes will increase our understanding of the role of the chromatin-based mechanisms in the long-term maintenance of cardiac gene expression and function.

Implication of ASXL2 in human heart disease

Heart diseases have been associated with mutations in a variety of genes that encode transcription factors, cytoskeleton components and signaling molecules. However, the molecular basis for a large number of heart diseases remains elusive. While genetic factors are certainly critical, epigenetic factors may be just as important. Epidemiological studies have led to the theory that many adult disorders, including cardiac disorders, may originate from fetal or early postnatal programming through epigenetic mechanisms [33]. Epigenetic programming can be influenced by maternal and infantile diet, by environmental factors, by mutations in genes that encode epigenetic regulators, or by the combinatorial effect of the above. We showed that ASXL2 is down-regulated in the hearts of patients with ischemic or idiopathic dilated cardiomyopathy. Down-regulation of ASXL2 may be a mere consequence of these heart conditions and may not play any active role; in this case, it may serve as a molecular marker in diagnosis. Alternatively, it may actively contribute to the onset of development of these conditions. This would raise the question of whether restoring ASXL2 function, or boosting the activity of histone modification enzymes that are regulated by ASXL2, can prevent or hinder disease progression. In-depth studies of mouse *Asxl2* will provide valuable insight on the diagnostic and/or therapeutic value of human ASXL2 in heart disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- The chromatin factor *Asxl2* is required for the maintenance of ventricular function.
- Ventricular dysfunction in *Asxl2*$^{-/-}$ hearts do not induce hypertrophy.
- *Asxl2* is required for repression of β-MHC.
- *Asxl2* directly binds to β-MHC promoters and co-localizes with the histone methyltransferse Ezh2.
- Human *ASXL2* is down-regulated in ischemic or idiopathic dilated cardiomyopathy patients.

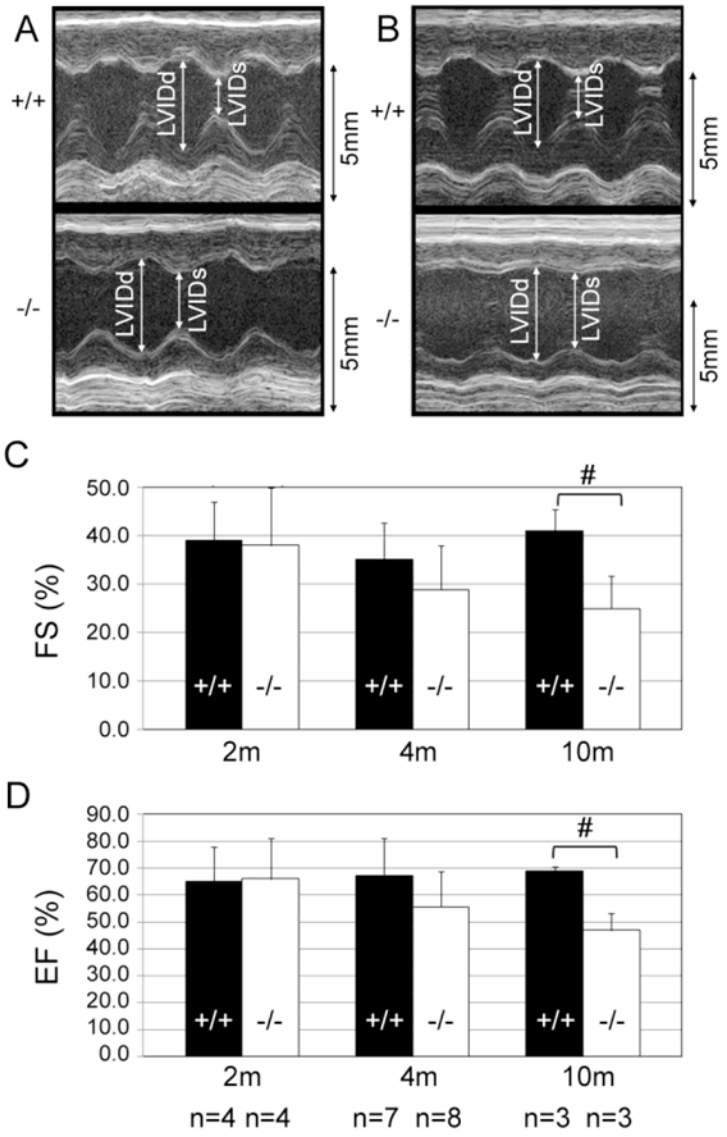

FIGURE 1. Evaluation of systolic function in *Asxl2*$^{-/-}$ mice and wild-type littermates

(A, B) Representative M-mode images of wild-type and *Asxl2*$^{-/-}$ hearts at 4 months (A) and 10 months (B), respectively. (C, D) Fractional shortening (C) and ejection fraction (D) in wild-type and *Asxl2*$^{-/-}$ hearts at 2 months, 4 months and 10 months. The numbers (n) of animals examined for each genotype at each time point are indicated at the bottom. The error bars represent standard deviations. # $p < 0.05$.

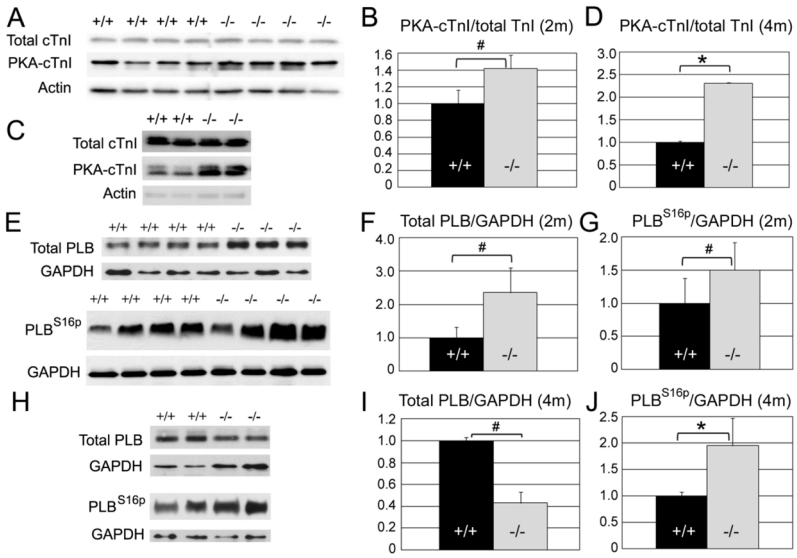

FIGURE 2. Expression and phosphorylation of cTnI and PLB in wild-type and *Asxl2*$^{-/-}$ hearts

(AD) Western blot analysis of total and PKA-phosphorylated cTnI at 2 months (A, B) and 4 months (C, D). The ratio of PKA phosphorylated cTnI to total cTnI was quantified by densitometry and shown in C (2m) and D (4m). (E–J) Western blot analysis of total and phosphorylated PLB at 2 months (E–G) and 4 months (H–J). F and G show the levels of total PLB and PLB^{S16p} normalized to that of GAPDH at 2 months. I and J show the levels of total PLB and PLB^{S16p} normalized to that of GAPDH at 4 months. * $p < 0.01$; # $p < 0.05$.

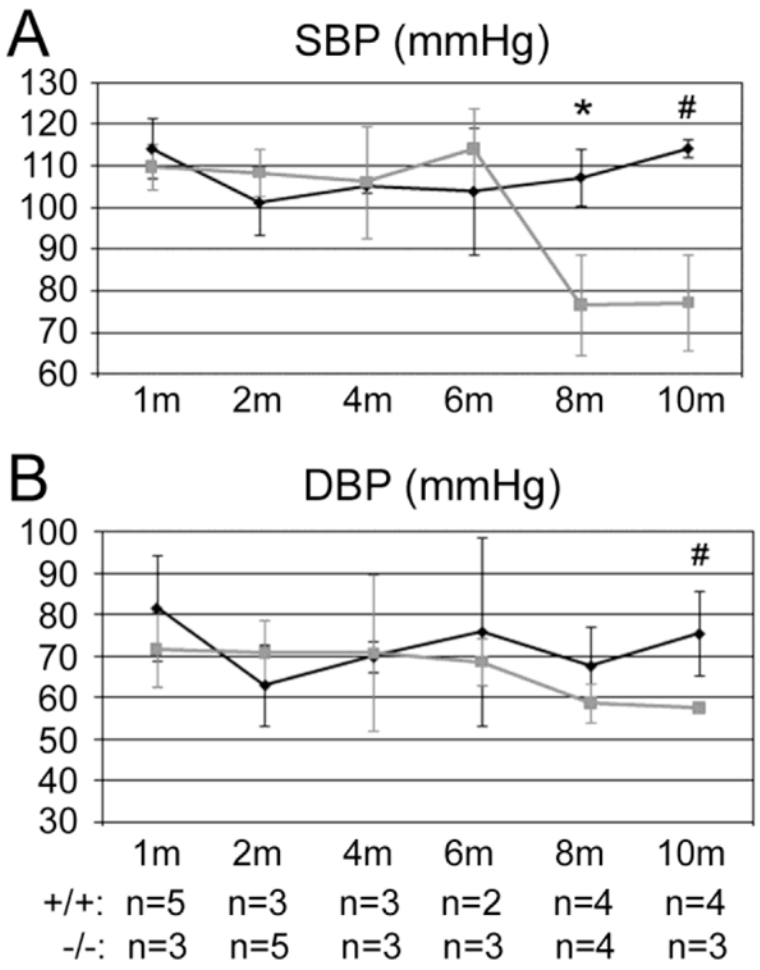

FIGURE 3. Blood pressure in wild-type (black lines) and *Asxl2*$^{-/-}$ mice (gray lines)

(A) Systolic blood pressure. (B) Diastolic blood pressure. The error bars represent standard deviations. * $p < 0.01$; # $p < 0.05$.

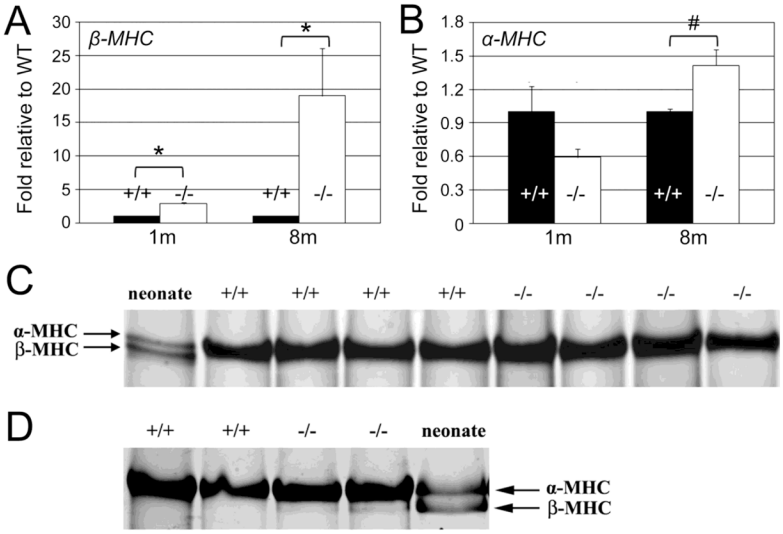

FIGURE 4. ***Asxl2*$^{-/-}$ heart exhibited progressive de-repression of *β-MHC***

(A) Real-time RT-PCR analysis of *β-MHC* transcripts in wild-type and *Asxl2*$^{-/-}$ hearts. De-repression of *β-MHC* became more pronounced with age. * $p < 0.01$. (B) Real-time RT-PCR analysis of *α-MHC* transcripts in wild-type and *Asxl2*$^{-/-}$ hearts. # $p < 0.05$. (C, D) High-resolution SDS-PAGE analysis of α- and β-MHC proteins in wild-type and *Asxl2*$^{-/-}$ hearts at 2 months (C) and 4 months (D). β-MHC protein became detectable in *Asxl2*$^{-/-}$ heart extract at 4 months.

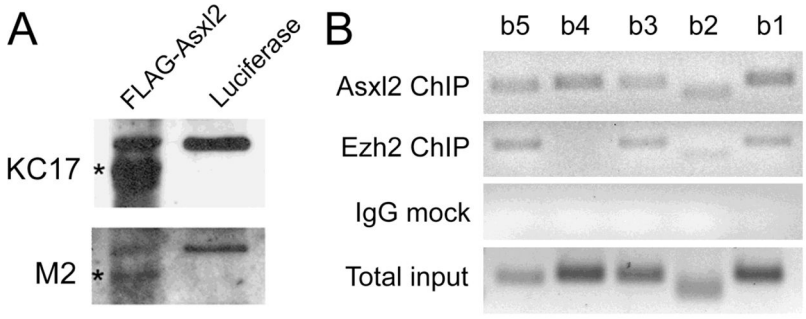

FIGURE 5. Asxl2 binds to β-MHC promoter and co-localizes with Ezh2 histone methyltransferase

(A) The polyclonal antibody KC17 recognizes Asxl2 on Western blot. FLAG-tagged Asxl2 or luciferase was expressed using rabbit reticulocyte in vitro transcription/translation system and probed with KC17 anti-Asxl2 antibody (top panel) or M2 anti-FLAG antibody (bottom panel). Both antibodies detected a band corresponding to the molecular weight of Asxl2 (asterisk) in the FLAG-Asxl2 translation mixture but not in the luciferase translation mixture. A non-specific band of higher molecular weight was detected in both mixtures and by both antidobies. (B) Chromatin immunoprecipitation assays of Asxl2 and Ezh2 enrichment at 5 conserved regions in the β-MHC promoter. Ezh2 and Asxl2 co-localize at 4 of the 5 regions analyzed.

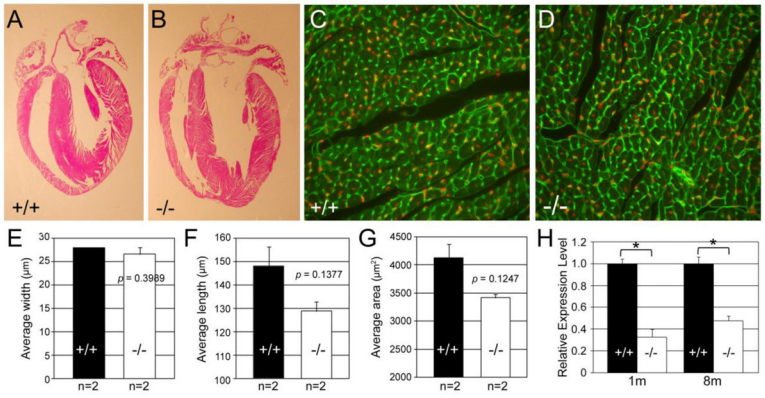

FIGURE 6. *Asxl2*$^{-/-}$ hearts did not develop hypertrophy

(A, B) Wild-type and *Asxl2*$^{-/-}$ heart sections were stained with hematoxylin and eosin. Scale bar: 1mm. (C, D) WGA stainings (green) of heart sections. Nuclei were stained with DAPI and pseudocolored in red. Both fluorescent images are shown at the same magnification. (E–G) Measurement of the average width (E), length (F) and area (G) of cardiomyocytes isolated from wild-type and *Asxl2*$^{-/-}$ hearts. (H) Real-time RT-PCR analysis of *Nppa* transcription at 1 month and 8 months. * $p < 0.01$.

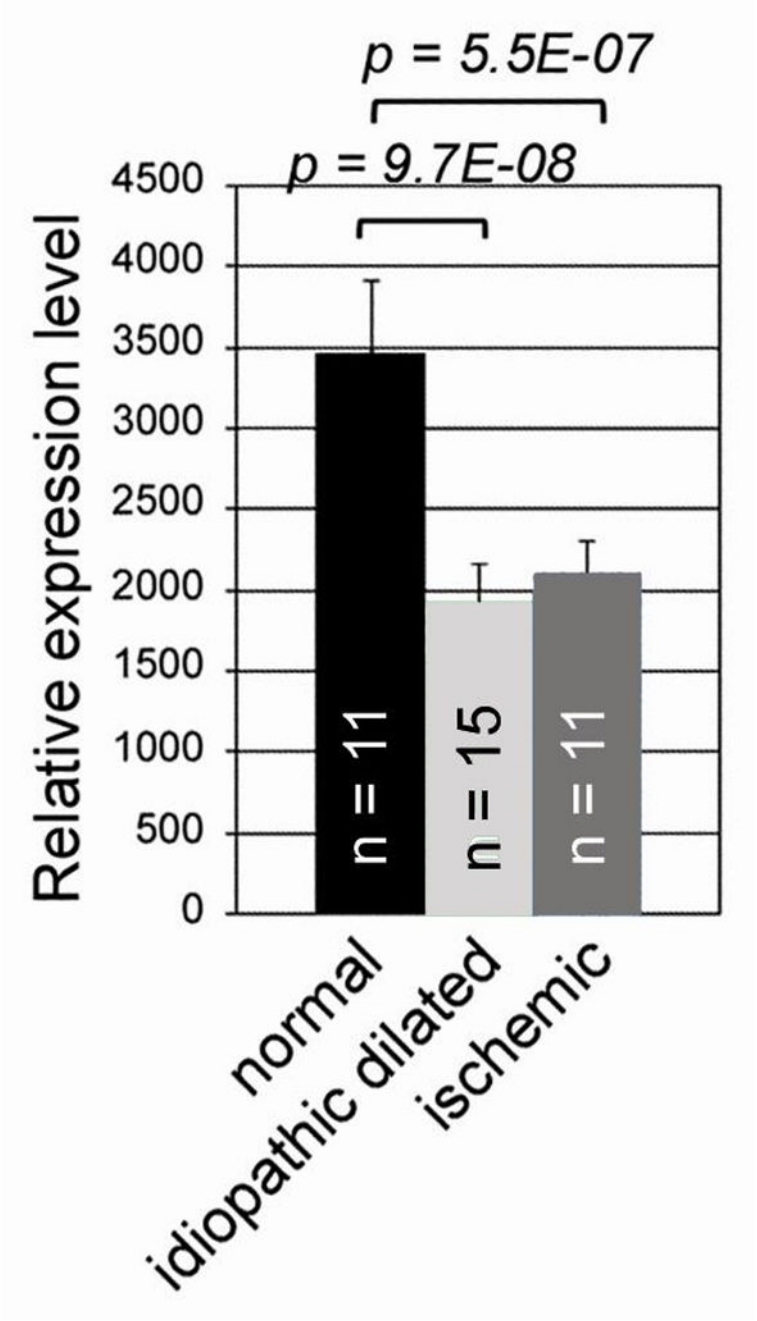

FIGURE 7. ***ASXL2*** **expression in human cardiomyopathy patients**

Graphs were made using microarray data generated by the CardioGenomics project. Expression levels of *ASXL2* in the hearts of normal people (organ donors whose heart were healthy but could not be used in transplant), patients with idiopathic dilated cardiomyopathy, and patients with ischemic cardiomyopathy. Data shown in the graph represent probe set 218658_at, which gave the higher signal intensity. Probe set 1555266_a_at had lower signal intensity but similar trends.