

Gene regulatory programs underlying regenerative
competence and stress resilience in the naked mole-rat
heart



Doctoral thesis
for
the award of the doctoral degree
of the Faculty of Mathematics and Natural Sciences
of the University of Cologne

submitted by

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2026

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Abstract

Unlike certain vertebrates such as zebrafish, which retain robust cardiac regeneration throughout life, mammalian hearts have only a transient regenerative window that is lost during postnatal maturation. This process is accompanied by cardiomyocyte cell-cycle exit, metabolic reprogramming, and a marked decline in proliferative capacity. The naked mole-rat (NMR; *Heterocephalus glaber*) displays unique cardiac features, including resistance to hypoxia and ischemic injury, retention of fetal-like metabolic characteristics, and regenerative ability following myocardial injury. However, the molecular basis of these distinctive cardiac traits remains poorly understood.

In this thesis, we investigated the genetic and epigenetic mechanisms underlying the unique cardiac phenotype of NMR using integrative multi-omics analyses and cross-species comparisons with mouse hearts. Our results revealed reduced postnatal remodeling in NMR hearts, accompanied by the persistence of neonatal-like molecular programs. In addition, integrative analysis of transcriptomic and histone ChIP-seq data further identified differentially regulated genes (DRGs) associated with coordinated changes in cis-regulatory elements. These regulatory programs drive cell-cycle-related processes, cytoskeletal remodeling, and metabolic rewiring characterized by reduced fatty acid oxidation (FAO), enhanced glycogen-dependent glycolysis, and redirection of fatty acid utilization from FAO and lipid storage toward phospholipid anabolism. Motif enrichment and DNA footprinting analyses identified estrogen-related receptors (ERRs) as key regulators of this metabolic rewiring. Furthermore, ERBB2 was identified as a neonatal-like regenerative driver that is maintained at the transcriptional, epigenetic, and protein levels in adult NMR hearts but is absent in adult mouse hearts. ERBB2 phosphorylation was further increased following cardiac injury, suggesting sustained ERBB2 signaling that may contribute to the regenerative competence of adult NMR hearts. Under hypoxic conditions, adult NMR hearts adopted an even more neonatal-like transcriptional profile, accompanied by activation of regeneration- and extracellular matrix-related genes, potentially through reactivation of bivalent chromatin states.

Collectively, our findings uncover the regulatory basis of the unique cardiac phenotype and stress adaptation of the adult NMR heart, providing mechanistic insights into evolutionary cardiac adaptation and establishing a foundation for strategies to promote cardiac regeneration in adult mammals.

Zusammenfassung

Im Gegensatz zu bestimmten Wirbeltieren wie dem Zebrafisch, die während ihres gesamten Lebens eine ausgeprägte kardiale Regenerationsfähigkeit aufrechterhalten, verfügen Säugetierherzen nur über ein kurzes regeneratives Zeitfenster in den ersten sieben Tagen nach der Geburt, das im Verlauf der postnatalen Reifung zunehmend verloren geht. Der Nacktmull (*Heterocephalus glaber*) weist einzigartige kardiale Eigenschaften auf, darunter eine hohe Resistenz gegenüber Hypoxie und ischämischen Schäden, die Aufrechterhaltung fetaler metabolischer Merkmale sowie eine Regenerationsfähigkeit nach myokardialer Schädigung. Die molekularen Mechanismen, die diesen besonderen kardialen Eigenschaften zugrunde liegen, sind jedoch bislang nur unzureichend verstanden.

In dieser Arbeit untersuchten wir die genetischen und epigenetischen Mechanismen, die dem einzigartigen kardialen Phänotyp des Nacktmulls zugrunde liegen, mithilfe integrativer Multi-Omics-Analysen und vergleichender Untersuchungen mit Mausherzen. Unsere Ergebnisse zeigten eine abgeschwächte postnatale Reifung des Nacktmull-Herzens, begleitet von der Persistenz entwicklungsassoziierter molekularer Programme. Darüber hinaus identifizierte die integrative Analyse von Transkriptom- und Histon-ChIP-seq-Daten differentiell regulierte Gene (DRGs), deren Expressionsänderungen mit koordinierten Veränderungen cis-regulatorischer Elemente assoziiert sind. Diese regulatorischen Programme fördern zellzyklusassoziierte Prozesse, die Umstrukturierung des Zytoskeletts sowie eine metabolische Umprogrammierung mit verringerter Fettsäureoxidation, verstärkter glykogenabhängiger Glykolyse und bevorzugter Phospholipidbiosynthese. Motivanreicherungs- und DNA-Footprinting-Analysen identifizierten Transkriptionsfaktoren der Estrogen-Related-Receptor-(ERR)-Familie als zentrale Regulatoren dieser metabolischen Umprogrammierung. Darüber hinaus wurde ERBB2 als entwicklungsassoziierter Regenerationstreiber identifiziert, der im adulten Nacktmull-Herz auf transkriptioneller, epigenetischer und Proteinebene erhalten bleibt und nach kardialer Schädigung verstärkt phosphoryliert wird. Unter hypoxischen Bedingungen zeigten adulte Nacktmull-Herzen ein stärker entwicklungsähnliches Transkriptionsprofil mit der Aktivierung regenerationsassoziierter Gene, möglicherweise infolge der Reaktivierung bivalenter Chromatinzustände.

Zusammenfassend liefern unsere Ergebnisse neue Einblicke in die regulatorischen Grundlagen des einzigartigen kardialen Phänotyps sowie der Stressanpassung des adulten Nacktmull-Herzens. Sie tragen zum Verständnis der evolutionären Anpassung des Herzens bei und bilden eine Grundlage für die Entwicklung neuer Strategien zur Förderung der kardialen Regeneration im adulten Säugetierherz.

1 Introduction

1.1 Unusual physiology in naked mole-rat

The naked mole-rat (NMR; *Heterocephalus glaber*) is a subterranean rodent species that inhabits the arid regions of East Africa, primarily found in Somalia, Ethiopia, and Kenya. Their underground lifestyle and extreme environmental conditions have led to the development of unique morphological traits and remarkable physiological adaptations that enable their survival in such harsh habitats[1–3]. These naturally evolved characteristics make them a fascinating model for scientific research, attracting interest from scientists across various fields.

1.1.1 Highly organized eusocial structure

NMRs exhibit a unique eusocial structure similar to that observed in certain insects, such as ants and bees[4]. Jarvis first described this eusocial system in detail and provided compelling evidence that NMRs represent the first confirmed eusocial mammals[4]. In a typical colony comprising up to 300 individuals, reproduction is dominated by a single breeding queen together with one to three breeding males[5] (Fig.1), whereas all remaining colony members are reproductively suppressed unless disruptions in social hierarchy occur.

Within the colony, non-reproductive individuals engage in a cooperative division of labor essential for survival in the dark, hypoxic subterranean environment[4]. Smaller and younger individuals typically act as frequent workers, responsible for tunnel excavation, food collection, and pup grooming (Fig.1). Larger individuals function as infrequent workers or soldiers, defending the colony against predators and supporting structurally demanding tasks[6] (Fig.1). These roles are not fixed, individuals may shift among tasks as they age or as colony needs change, demonstrating the dynamic and adaptable nature of the NMR social system[7]. The organization of these roles is highly plastic and can change with age, experience, or colony needs. The extensive cooperation and reproductive specialization provide substantial adaptive advantages in their low-oxygen, resource-scarce environment, supporting the persistence of large, stable colonies underground.

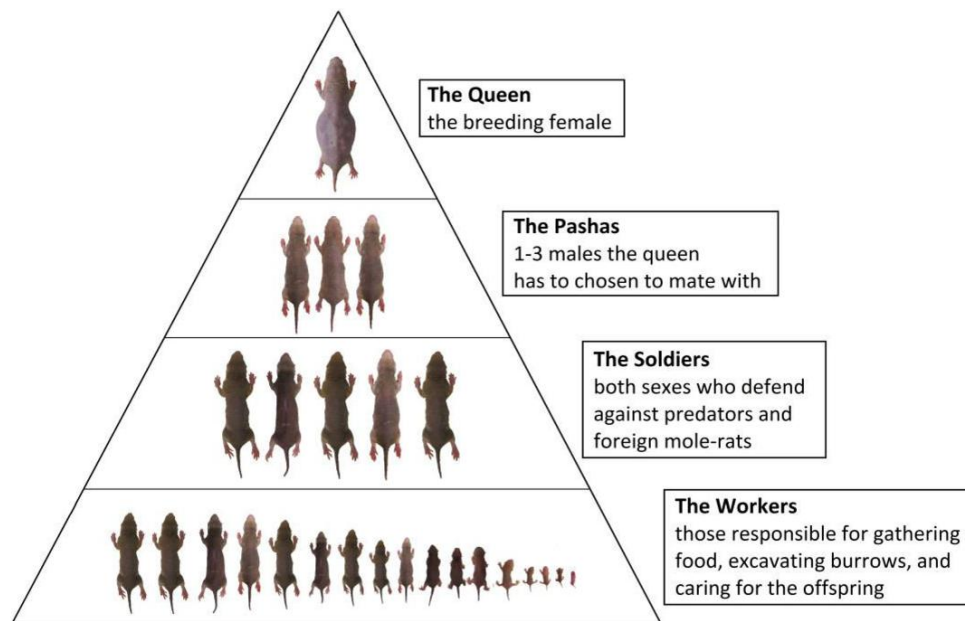


Fig. 1. Social hierarchy and division of labor in a naked mole-rat colony.

Naked mole-rats exhibit a eusocial colony structure organized into distinct social castes. A single breeding female ("the queen") monopolizes reproduction and mates with one to three selected breeding males ("the pashas"). Non-breeding colony members are divided into soldiers and workers. Soldiers of both sexes defend the colony against predators and foreign mole-rats, whereas workers are responsible for foraging, tunnel excavation, nest maintenance, and caring for offspring. Colony size can reach up to approximately 300 individuals.

1.1.2 Extreme tolerance to hypoxia, hypercapnia and pain

Not only can hundreds of NMRs live together within a single burrow, but under laboratory conditions, they have also been observed frequently piling on top of one another[8]. Living in poorly ventilated underground burrows, along with their large colony sizes and densely crowded social environments, exposes NMRs to low oxygen (O_2) and high carbon dioxide (CO_2) conditions. For most surface-dwelling mammals, O_2 depletion is typically fatal, and excessive CO_2 can lead to acidosis, making survival extremely challenging under severe conditions. In contrast, through long-term evolutionary adaptation, NMRs have developed exceptional tolerance to both hypoxia and hypercapnia, allowing them to survive and maintain essential physiological functions in these environments[3, 9].

Previous studies have demonstrated the extraordinary hypoxia tolerance of NMRs. When comparing the survival rates of NMRs and mice of similar body size under different hypoxic conditions, all NMRs survived for up to five hours under 5% O_2

experimental conditions, whereas the mice died within 15 minutes under the same conditions[10] (Fig.2). Even under extreme oxygen-deprived conditions (0%), NMRs can still survive for at least 18 minutes and recover without noticeable behavioral abnormalities[10] (Fig.2). Although such extreme conditions are unlikely to be encountered in their natural habitat, these findings clearly indicate that NMRs possess intrinsic physiological mechanisms that support survival during profound oxygen deprivation.

At the organismal level, hypoxia tolerance in NMRs is supported by coordinated metabolic and thermoregulatory responses. Other mammals primarily maintain a constant body temperature through physiological regulation, a process that requires significant energy consumption[11]. In contrast, NMRs seek suitable ambient temperatures by moving to different depths within their tunnels [12]. Moreover, by huddling together, they can maintain a stable body temperature. Furthermore, NMRs markedly reduce spontaneous activity and suppress non-shivering thermogenesis, thereby lowering their body temperature to a level close to that of environment. This metabolic state substantially lowers whole-body energy expenditure and oxygen demand, thereby extending survival under hypoxic stress[9, 13]. Notably, unlike many hypoxia-tolerant vertebrates that completely enter torpor or coma-like states, NMRs remain conscious and responsive despite this metabolic downregulation, reflecting a unique balance between energy conservation and functional activity[13, 14]. To meet the demands of low oxygen levels, metabolic rate, and energy expenditure, metabolism in NMRs has been rewired to utilize a fructose-based glycolytic pathway to fuel major tissues and organs[10].

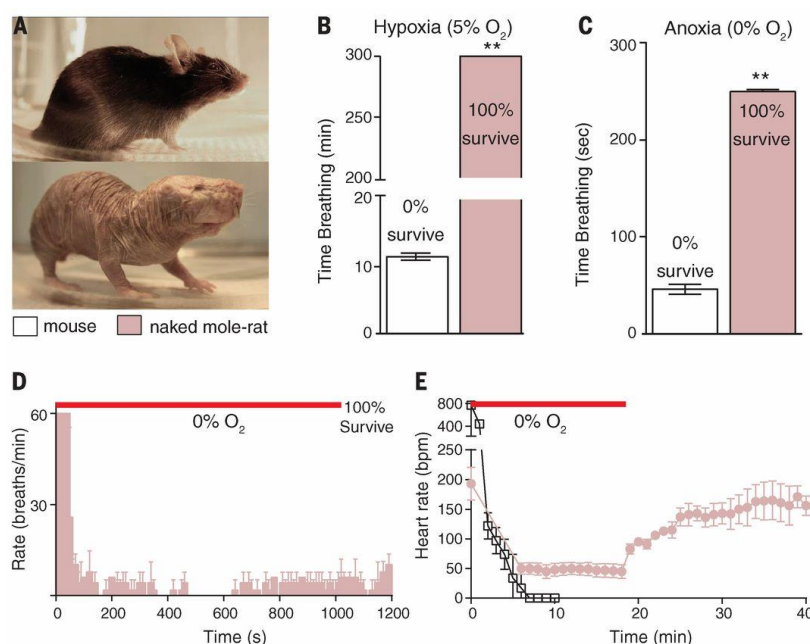


Fig. 2. Exceptional hypoxia and anoxia tolerance in naked mole-rats.

(A) Representative images of a mouse (top) and a naked mole-rat (bottom). (B and C) Survival under severe hypoxia (5% O_2) (B) and complete oxygen deprivation (0% O_2 , anoxia) (C). Naked mole-rats remained alive for prolonged exposure, whereas mice failed to survive under the same conditions. Respiratory (D) and Heart rate (E) changes during anoxic exposure.

In most mammals, elevated CO_2 levels cause acidosis, pulmonary edema, and significant physiological distress. However, NMRs exhibit an exceptional tolerance to hypercapnia. They do not develop acidosis when exposed to CO_2 concentration below 10%, whereas mice can experience acidosis at levels as low as 1% CO_2 . Remarkably, NMRs show no signs of pulmonary edema even under extreme conditions with 50% CO_2 exposure[10]. High concentrations of CO_2 can lead to acidosis, which in turn causes pain. However, NMRs also have an extraordinary capacity to tolerate pain. When injected with irritants such as acid or capsaicin, NMRs do not exhibit any pain response, whereas mice show a strong pain response under the same conditions[15]. Consequently, presence of all these remarkable physiological traits together enables NMRs to achieve exceptional adaptability to such a harsh underground environment.

1.1.3 Exceptional longevity, negligible senescence, and cancer resistance

In addition to possessing a suite of remarkable physiological traits, NMRs have become renowned as an important model organism in biomedical research because they also exhibit longevity and resistance to aging and cancer[16, 17]. NMRs can live for more than 35 years, whereas other rodents of similar body size, like mice, usually live for less than 4 years[18]. This represents a lifespan at least 5-10 times longer than predicted by allometric scaling based on body mass[17]. Intriguingly, in contrast to the typical aging patterns observed in other mammals, NMRs do not exhibit a significant increase in mortality with age. This defies the Gompertz law of aging, which governs most mammalian species, instead, NMR mortality remains essentially constant across their lifespan, a hallmark of negligible senescence[19, 20]. Usually, certain physiological functions decline with age, such as reproductive capacity, metabolic stability, and cardiovascular performance, yet these capabilities remain well preserved in NMRs even in advanced age[1, 16, 21, 22].

Based on these characteristics of NMRs, many studies have been conducted to explore genetic mechanisms, thereby providing insights into improving human health. In addition, a variety of molecular and cellular mechanisms have been identified that are uniquely adapted in NMRs and are thought to contribute to their remarkable phenotypes. NMRs maintain highly efficient proteostasis, characterized by elevated proteasomal activity, reduced protein aggregation, and increased stability of cytosolic and mitochondrial proteins[1, 23, 24]. NMRs exhibit an enhanced capacity to maintain genome stability. Their cells showed increased resistance to oxidative damage and maintained robust DNA repair capacity, which together limit the accumulation of cellular damage typically associated with aging[17, 24, 25]. This enhanced maintenance is also supported by unique genomic features, such as increased translational fidelity and specialized chaperone networks[26–28].

Importantly, this extended lifespan is accompanied not only by an absence of the typical age-related physiological decline but also by a remarkably low incidence of spontaneous malignancies. Early studies reported an almost complete absence of spontaneous tumors in large captive populations of NMRs, even at very advanced ages[29, 30]. At the cellular level, NMR fibroblasts display an unusual form of contact inhibition, termed early contact inhibition, which prevents uncontrolled cell proliferation at lower cell densities than observed in mouse or human cells[31] (Fig.3). Furthermore,

NMR cells secrete an exceptionally high-molecular-mass hyaluronan (HMM-HA) that forms a dense, anti-cancer extracellular matrix[32] (Fig.3). HMM-HA activates specific cell surface receptors and downstream signaling pathways that reinforce cell cycle arrest and suppress oncogenic transformation. Genetic or enzymatic disruption of HMM-HA synthesis significantly increases the susceptibility of NMR cells to malignant transformation, highlighting its central role in cancer resistance[33]. In addition, comparative genomic and transcriptomic studies have also identified distinctive features in genes involved in cell cycle regulation, DNA damage response, and genome stability, further supporting a genetically reinforced tumor-suppressive landscape in NMRs[34–37].

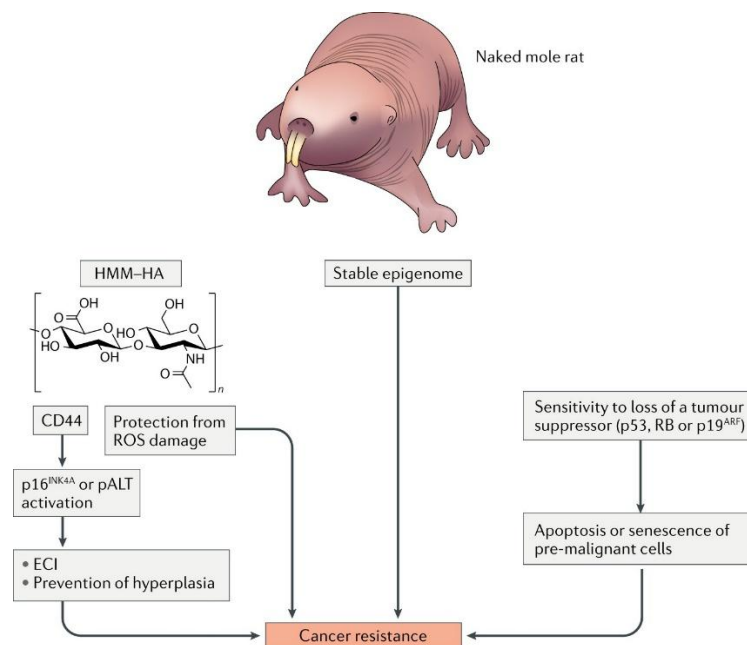


Fig. 3. Proposed mechanisms underlying cancer resistance in naked mole-rats.

Naked mole-rats possess multiple protective mechanisms that contribute to their remarkable resistance to tumor formation. High-molecular-mass hyaluronan (HMM-HA) interacts with the CD44 signaling pathway and promotes activation of tumor-suppressive programs, including p16^{INK4a} and alternative reading frame (pALT), leading to enhanced early contact inhibition (ECI) and suppression of uncontrolled cell proliferation. In parallel, HMM-HA and other cellular adaptations provide protection against reactive oxygen species (ROS)-induced damage. Naked mole-rats also exhibit enhanced sensitivity to the loss of major tumor suppressors, resulting in apoptosis or senescence of pre-malignant cells. Together with maintenance of a stable epigenome, these mechanisms collectively contribute to the exceptional cancer resistance observed in naked mole-rats.

1.2 Heart regeneration

Cardiovascular diseases, including heart attacks, remain the leading cause of death worldwide, largely because the adult mammalian heart has only a limited capacity for self-repair. Investigating regenerative mechanisms across developmental stages and in species capable of heart regeneration can provide important insights into the molecular basis of cardiac repair, ultimately contributing to the development of new therapeutic strategies for human cardiovascular diseases. Many vertebrates retain a strong capacity for cardiac regeneration during their whole lives, for example, zebrafish, axolotl, and newts exhibit remarkable heart regenerative abilities[38–40]. However, in mammals, this capacity is lost shortly after birth[41, 42]. Thus, making the study of regenerative mechanisms in these regenerative species particularly valuable for understanding why and how heart regeneration becomes limited in mammals.

1.2.1 Cell cycle

In adult mammals, the limited proliferative capacity of cardiomyocytes (CMs) restricts heart repair after injury. Shortly after birth, mammalian CMs lose their ability to proliferate as they exit the cell cycle and undergo differentiation, accompanied by a rapid transition from CM hyperplasia to hypertrophy[43]. In contrast, zebrafish can fully regenerate the heart even after resection of approximately 20% of the ventricle. This regenerative process is primarily driven by pre-existing CMs, which re-enter the cell cycle, proliferate, and replace the lost cardiac tissue[44].

In adult mammalian CMs, polyploidization occurs during the cell cycle[45]. Under normal cell cycle progression, a parent cell prepares for DNA replication during the G1 phase, completes DNA replication in the S phase, and grows during the G2 phase, followed by karyokinesis and cytokinesis in the M phase, ultimately dividing into two daughter cells (Fig. 4). However, in adult mammalian CMs, cells typically withdraw from the cell cycle after completing DNA replication during S phase without undergoing karyokinesis, resulting in the formation of mononuclear polyploid CMs (Fig. 4). In other cases, cells undergo karyokinesis but exit the cycle before cytokinesis, resulting in multinucleated cells (Fig. 4).

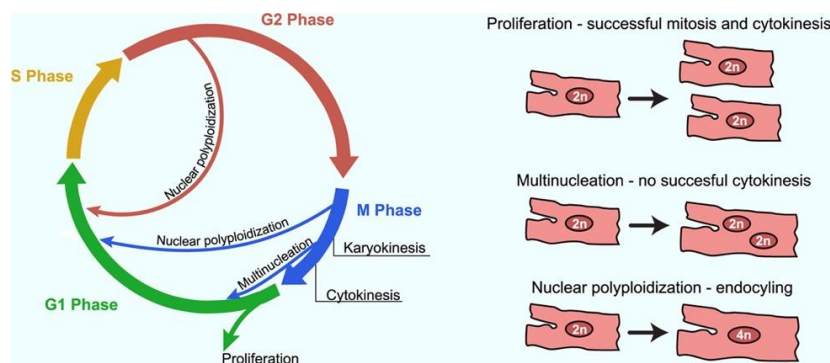


Fig. 4. Cell cycle progression and polyploidization processes in mammalian cardiomyocytes.

Schematic overview of cardiomyocyte cell-cycle progression and the distinct cellular outcomes that occur during postnatal cardiac maturation. Following DNA synthesis during S phase and progression through G2 and M phases, cardiomyocytes can undergo complete cell division with successful mitosis and cytokinesis, resulting in proliferation and generation of two diploid daughter cells. Alternatively, incomplete cytokinesis can produce multinucleated cardiomyocytes, whereas endocycling without proper karyokinesis leads to nuclear polyploidization and increased DNA content within a single nucleus. These processes contribute to the postnatal decline in cardiomyocyte proliferative capacity in mammalian hearts.

A significant distinction between non-proliferative and fully proliferative CMs lies in their DNA content[45]. In species such as zebrafish and newts, CMs remain mononucleated and diploid throughout their entire lifespan[39, 46]. Similarly, during embryonic development in mammals, including mice and humans, all CMs are mononucleated and diploid[47, 48]. However, in postnatal mammals, most CMs become polyploid shortly after birth. In mice, the loss of cardiac regenerative capacity occurs on postnatal day 7, coinciding with the onset of CM polyploidization[47]. Moreover, the proportion of mononuclear diploid CMs is positively correlated with the extent of cardiac regeneration following injury[49]. Increasing the proportion of polyploid CMs has been shown to inhibit both CM proliferation and heart regeneration[50]. Therefore, in adult mammals, strategies that mobilize the existing diploid CM population may promote cardiac regenerative capacity.

Recent studies have shown that the inactivation or overexpression of certain genes can alter the CM cell cycle, thereby affecting the proportion of polyploid cells in the heart[50, 51]. For example, overexpression of CCNG1 (Cyclin G1) and CCND1 (Cyclin D1) promotes DNA synthesis in mouse CM but inhibits cytokinesis, resulting in an

increased number of binucleated CMs[52, 53]. Knockout of *Tnni3k* leads to an increased proportion of mononucleated diploid CMs and enhances CM proliferation after cardiac injury in adult mice[49]. Beyond intrinsic cell cycle regulation, specific signaling pathways can also modulate cell cycle activity and cytokinesis in CMs. Notably, constitutive activation of *ErbB2* markedly enhances cell cycle progression, mitosis, and cytokinesis, leading to an increased proportion of mononucleated CMs and promoting dedifferentiation and proliferation[54].

1.2.2 Sarcomere structure

CMs are the predominant cell type in the heart, and sarcomeres are the fundamental contractile units of striated muscle, including CMs. Each sarcomere extends from one Z-disc to the next. Cardiac muscle contraction is achieved through the interaction between thin and thick filaments, which are arranged in parallel within the sarcomere (Fig. 5). The sarcomere is composed of highly organized structural regions, including the Z-disc, I-band, A-band, H-zone, and M-line, where contractile proteins are arranged in a precise and repetitive pattern[55]. Thin filaments are primarily composed of actin together with the regulatory proteins troponin and tropomyosin (Fig. 5). Thick filaments consist mainly of myosin molecules and are associated with structural and regulatory proteins such as titin and myosin-binding protein C, which contribute to sarcomere stability, elasticity, and contractile function (Fig. 5). The coordinated interaction of these structural proteins enables the precise mechanical performance of the heart, and disruptions in sarcomere organization can lead to impaired cardiac function and disease.

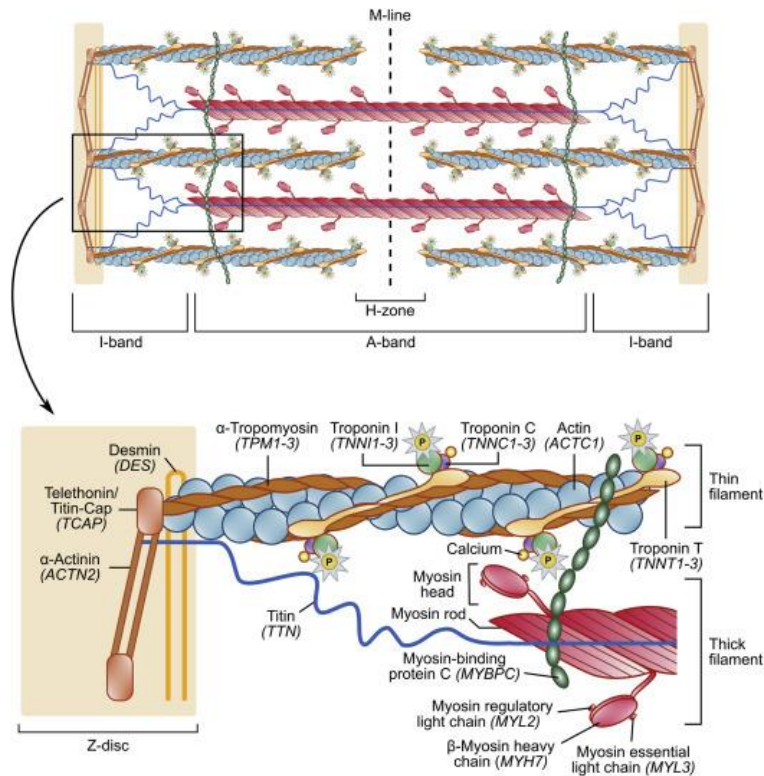


Fig. 5 Structural organization of the cardiac sarcomere and its major molecular composition.

(Top) Schematic representation of the sarcomere, the fundamental contractile unit of cardiomyocytes. The sarcomere is organized into distinct structural regions, including the Z-disc, I-band, A-band, H-zone, and M-line. Thin filaments are mainly composed of actin together with the regulatory proteins troponin and tropomyosin, which mediate calcium-dependent contraction. Thick filaments primarily consist of myosin molecules and are associated with accessory proteins such as myosin-binding protein C and titin, which contribute to sarcomere stability, elasticity, and force generation. The enlarged panel highlights the molecular organization of the Z-disc and the interactions among major sarcomeric proteins involved in cardiac contraction and structural integrity. (Bottom) Representation of the major proteins in the cardiac sarcomere.

Sarcomeres are essential not only for the contractile function of CMs but also play a pivotal role in ability of heart to regenerate. During development and in regenerative species such as neonatal mice and zebrafish, CMs are characterized by less mature, more disorganized sarcomeres, which are thought to facilitate cell cycle re-entry and proliferation[41, 44]. In contrast, mature mammalian CMs develop highly organized and tightly packed sarcomeres shortly after birth, coinciding with cell-cycle withdrawal and loss of regenerative capacity[41]. In zebrafish, CMs in uninjured myocardium show well-aligned sarcomeres and are clearly defined by Z-discs. However, during

regeneration, the spacing between CMs increases, and their sarcomeres become disorganized and lose their Z-disc integrity. This suggests that the structural maturation of sarcomeres acts as a barrier to CM proliferation.

During mammalian heart development, the sarcomere structure undergoes significant remodeling, transitioning from the embryonic/neonatal to the adult form. This structural maturation is accompanied by the isoform switching of several key sarcomere components. In mice, the myosin heavy chain predominantly expressed during embryonic development is *Myh7* (Myosin heavy chain 7), which transitions to *Myh6* (Myosin heavy chain 6) expression in adulthood[56]. Conversely, in humans, *Myh6* is mainly expressed during fetal development, with *Myh7* becoming the dominant isoform in adult hearts[57]. Beyond myosin heavy chains, other key sarcomeric proteins also undergo isoform transitions. For instance, *Tnni1* (Troponin I1, slow skeletal type), the fetal form of cardiac troponin I, is replaced by the cardiac-specific *Tnni3* (Troponin I3, cardiac type) during maturation[58, 59]. In humans, this transition from *Tnni1* to *Tnni3* is completed around 9 months after birth[58]. In addition to isoform switching, other mechanisms also contribute to sarcomere disassembly. Constitutively active *ErbB2* in mice also promotes disassembly of sarcomeric structure, a process associated with CM dedifferentiation[54]. In addition, epigenomic studies in adult zebrafish during heart regeneration have demonstrated that AP-1 contributes to chromatin remodeling and promotes sarcomere disassembly[60]. Notably, one of its target genes, *Fbxl22*, which located Z-disc and mediates proteasomal degradation of key sarcomeric proteins, can also promote sarcomere breakdown when overexpressed[60, 61].

1.2.3 Energy metabolism

Maturation of CMs is accompanied by profound structural and functional changes, including enhanced contractile capacity and progressive mitochondrial maturation[62, 63]. During this process, CMs undergo a major metabolic transition from predominantly glycolytic metabolism to fatty acid oxidation (FAO) and mitochondrial oxidative phosphorylation (OXPHOS) as the primary sources of ATP production[64, 65]. Although this metabolic shift enables the mature heart to meet its high energetic demands, it is also associated with the establishment of a barrier to CM proliferation and regeneration[66].

Recent studies have further demonstrated that reducing lipid-dependent energy metabolism and shifting CMs toward a more glycolytic metabolic state can promote proliferative and regenerative capacity. For example, activation of glycolytic regulators such as *Pkm2* promotes CM cell-cycle re-entry and improves cardiac regeneration following injury[67]. Similarly, inhibition of *Pdk4*, a negative regulator of glucose oxidation, enhances glycolytic metabolism and has been associated with increased CM proliferative capacity[66]. In contrast, suppression of mitochondrial oxidative metabolism and FAO can also promote regeneration. Inhibition of enzymes involved in FAO, including *Cpt1b*, promotes CM proliferation and cardiac repair after injury[68]. Moreover, inhibition of *Ppargc1a*, a key regulator of mitochondrial oxidative metabolism, enhances glycolytic flux and increases CM proliferation in response to cardiac damage[69].

Metabolic remodeling also contributes to regeneration through mechanisms beyond energy supply. Enhanced mitochondrial oxidative metabolism increases reactive oxygen species (ROS) production, which can induce DNA damage responses and reinforce CM cell-cycle arrest[64]. Glycolytic metabolism, by contrast, supports anabolic pathways required for cell proliferation and reduces oxidative stress[70]. In addition, metabolites generated from glucose and mitochondrial pathways can directly influence chromatin structure and transcriptional regulation through epigenetic modifications[71]. Therefore, metabolic state not only reflects developmental maturation but also actively regulates gene expression programs associated with regeneration[72].

Emerging evidence further indicates that metabolic pathways are tightly interconnected with major regenerative signaling networks, including Hippo-YAP, HIF and ERBB signaling[73–75]. These pathways coordinate cellular growth, mitochondrial function, glucose metabolism, and stress adaptation during tissue repair. Together, these findings support that metabolic remodeling is a central regulatory component of cardiac regeneration and may represent a promising therapeutic target for promoting heart repair in adult mammals.

1.2.4 Transcription factors

The heart is composed of multiple specialized cell types, and its development and function rely on precise spatial and temporal regulation of gene expression. To achieve

this, cardiac gene regulatory networks integrate extracellular signals with transcriptional programs that ensure genes are activated in the correct cell types and at appropriate developmental stages[76]. Transcription factors (TFs) lie at the core of these networks, acting as key regulators that coordinate CM proliferation, differentiation, maturation, and adaptive responses[77, 78].

TFs play particularly important roles in cardiac development and function. The Myocyte Enhancer Factor 2 (MEF2) family, which is encoded by four genes in vertebrates (Mef2a, Mef2b, Mef2c, and Mef2d), acts as a central regulator of CM differentiation and maturation and is required for both embryonic heart development and postnatal cardiac function[79]. MEF2 factors control the expression of genes involved in sarcomere organization, metabolism, and contractile performance, and their dysregulation is associated with impaired CM maturation and pathological remodeling[80–82].

In addition, ERRs (Estrogen-related receptors) have emerged as central regulators linking cardiac metabolism to CM maturation and regenerative capacity. The ERRs family consists of three members: Esrra, Esrrb, and Esrrg. Previous studies have shown that Esrra and Esrrg directly regulate extensive gene networks involved in FAO, oxidative phosphorylation, and mitochondrial biogenesis in CMs in postnatal hearts[83–85]. Importantly, CM-specific knockdown of Esrra and Esrrg demonstrated that ERRs play important roles in promoting heart metabolic maturation, contractile function and suppress fibroblast activation in postnatal heart development[86].

Another important group of regeneration-associated TFs is the AP-1 (activating protein-1) complex, primarily composed of Jun, Fos, ATF, and MAF subunits[87]. Importantly, functional evidence indicates that AP-1 activity is required for heart regeneration. In highly regenerative species like zebrafish, AP-1 is rapidly recruited to injury-induced enhancers to initiate a pro-regenerative gene program[60]. Moreover, in adult *Xenopus tropicalis*, loss of Fosl1, a core AP-1 component, severely impairs CM proliferation and myocardial regeneration following apex resection, leading to persistent fibrosis[88].

In addition to these TF families, the Hippo-YAP/TEAD axis provides a well-established example of how transcriptional regulators can directly promote mammalian cardiac regeneration. YAP activation in mouse CMs induces embryonic and proliferative gene programs and promotes cardiac regeneration after injury[89]. This pathway is

particularly relevant because YAP commonly acts through TEAD family transcription factors, linking extracellular mechanical and growth signals to gene expression programs that control CM proliferation[75, 90]. Although YAP/TEAD signaling is mechanistically distinct from MEF2, ERRs, and AP-1, it further supports the broader concept that TF networks can determine whether adult CMs remain post-mitotic or re-enter a regenerative state.

1.3 Unique feature of naked mole rat heart

1.3.1 Unique metabolic adaptations of the naked mole-rat heart

As we mentioned earlier, in most mammals, the primary metabolic pathways change as the environment shifts from hypoxic to normoxic conditions after birth. As a result, adult CMs predominantly rely on FAO to sustain ATP production, while carbohydrate utilization and glycogen storage are markedly reduced compared with the neonatal stage[91]. In contrast, NMR hearts exhibit a strikingly distinct metabolic profile that deviates from this canonical developmental trajectory. NMR hearts display an exceptional capacity to accumulate and retain carbohydrate-based energy reserves. Metabolomic analyses have revealed significantly elevated levels of glycogen and glucose-1-phosphate in NMR hearts compared not only with mice but also with closely related African mole-rats, indicating that this feature is specific to NMRs rather than a general trait of subterranean rodents[2, 92].

Consistent with these metabolic observations, our previous cardiac transcriptomic analyses further demonstrate a pronounced shift in substrate utilization at the gene expression level[93]. Compared with mouse hearts, genes upregulated in the NMR heart are significantly enriched for carbohydrate metabolism- and glycogen-related biological processes (Fig. 6A). In contrast, pathways associated with fatty acid β -oxidation are consistently enriched among downregulated genes (Fig. 6B and C). Unexpectedly, ultrastructural analyses provide additional insight into the unique nature of glycogen storage in the NMR heart. Transmission electron microscopy reveals that cardiac glycogen in NMRs is predominantly organized into large α -granules, a storage form typically characteristic in the liver, rather than the smaller β -granules commonly observed in mouse hearts (Fig. 6D). Compared with β -granules, α -granules are degraded into glucose at a slower rate, conferring greater stability to glycogen

stores[94]. This liver-like glycogen architecture suggests an enhanced capacity for long-term energy storage and rapid mobilization, potentially enabling the NMR heart to maintain ATP supply under fluctuating oxygen availability.

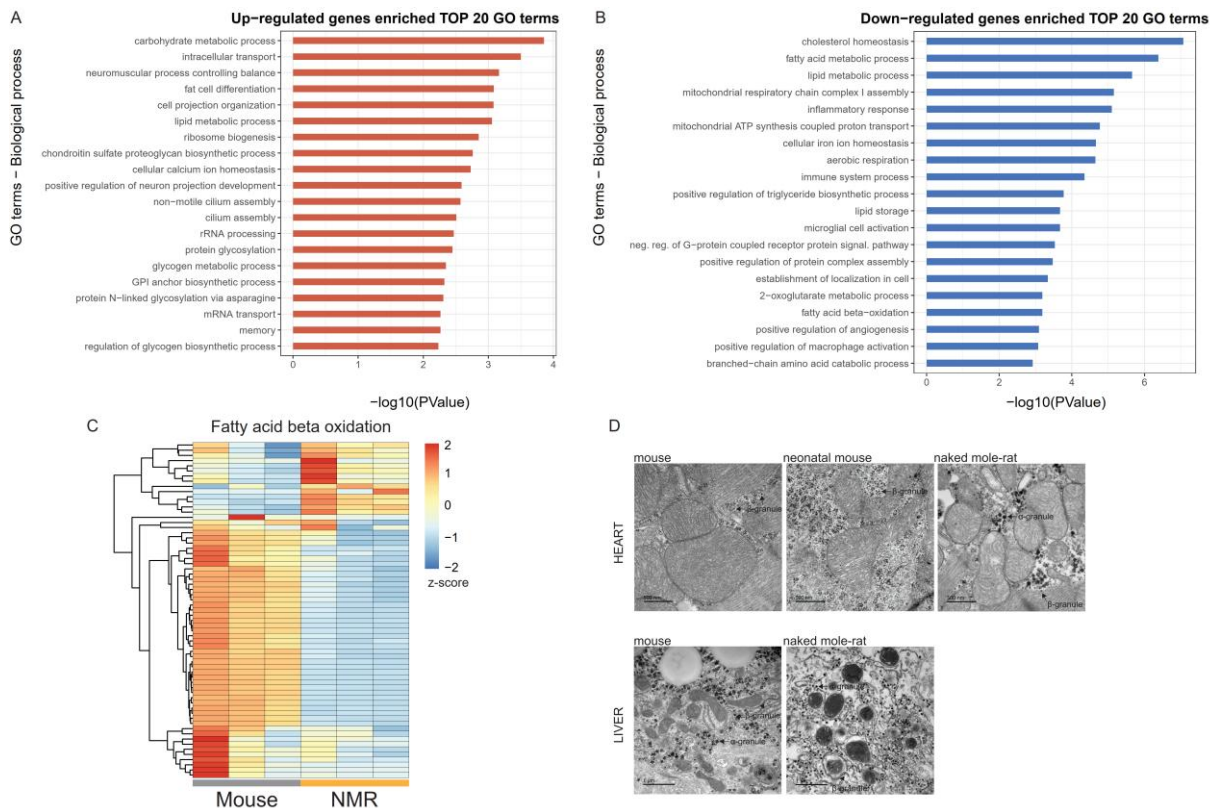


Fig. 6. Distinct carbohydrate-centered metabolic features of naked mole-rat heart.

(A) Gene ontology (GO) enrichment analysis of genes upregulated in adult naked mole-rat (NMR) hearts compared with adult mouse hearts, showing enrichment of carbohydrate metabolic processes and glycogen-related pathways. (B) GO enrichment analysis of genes downregulated in adult NMR hearts compared with adult mouse hearts, highlighting reduced enrichment of lipid metabolism, fatty acid metabolic processes, and mitochondrial respiratory functions. (C) Heatmap showing relative expression levels of genes involved in fatty acid β -oxidation in adult mouse and NMR hearts. Expression values are shown as row-scaled z-scores. (D) Representative transmission electron microscopy images of adult mouse, neonatal mouse, and adult NMR hearts, as well as adult mouse and NMR liver. NMR hearts display abundant glycogen granules resembling those observed in liver tissue. Glycogen particles are indicated as α -granules ($< 50\text{nm}$) and β -granules ($>50\text{nm}$). Heart images: $n = 3$ biological replicates, scale bar = 500 nm; liver images: $n = 2$ biological replicates, scale bar = 1 μm . Approximately 140-450 glycogen granules were quantified per sample.

Under hypoxic or anoxic stress, the metabolic plasticity of the NMR heart becomes even more pronounced. A key adaptive mechanism is the activation of a fructose-

driven glycolytic bypass. In NMRs, genes involved in fructose transport and metabolism, including GLUT5 (Slc2a5) and ketohexokinase (KHK), are highly expressed in cardiac tissue, and cardiac fructose levels rapidly increase under hypoxic conditions. By channeling fructose into glycolysis downstream of the phosphofructokinase (PFK) regulatory checkpoint, this pathway bypasses one of the major rate-limiting steps of canonical glycolysis, thereby enabling sustained glycolytic flux and ATP production even in the absence of oxygen[10]. This pathway, which is extremely rare in mammalian hearts, provides NMRs with a crucial survival advantage during prolonged oxygen deprivation. At the same time, glycogen reserves accumulated under resting conditions are rapidly mobilized and act synergistically with fructose metabolism to sustain energy supply in CMs.

1.3.2 Naked mole-rat heart resistant to ischemia

Ischemic injury remains a major cause of CMs loss and irreversible cardiac dysfunction in most mammals, as interruption of blood flow rapidly deprives the myocardium of oxygen and metabolic substrates[95]. Given the substantial energy demands of sustained contraction, ATP production in the adult mammalian heart relies predominantly on mitochondrial oxidative phosphorylation coupled with FAO[96]. Consequently, ischemia results in rapid ATP depletion, disruption of ionic homeostasis, accumulation of metabolic byproducts, and excessive oxidative stress upon reperfusion, ultimately leading to CM death and impaired cardiac function[97, 98].

In striking contrast, NMR hearts exhibit a remarkable resistance to ischemic injury. Previous studies using isolated hearts and ischemia-reperfusion models have consistently demonstrated that NMR hearts sustain significantly less structural and functional damage than mouse hearts under similar ischemic stress [2, 10]. Specifically, *in vivo* ischemia-reperfusion experiments performed in our lab revealed that NMR hearts exhibit reduced infarct size, attenuated tissue damage, and enhanced recovery of cardiac function following reperfusion compared with mouse hearts[2, 93]. These findings highlight intrinsic mechanisms in NMR hearts that resist ischemic injury and preserve cardiac function under stress.

One major contributor to this ischemia-resistant phenotype is the distinctive metabolic configuration of the NMR heart. As described above, NMR CMs rely less on FAO and instead maintain abundant carbohydrate reserves and a strong glycolytic capacity[92,

93]. Consistent with this, our previous metabolomic analyses under ischemic conditions revealed that NMR hearts exhibit a pronounced increase in glycogen-derived polysaccharides, indicating rapid mobilization of pre-existing glycogen stores[93]. This response is accompanied by elevated activity of glycogen-degrading enzymes, including amylase, thereby supporting the efficient conversion of stored glycogen into glycolytic substrates during ischemia[93]. As a result, glycolytic flux is maintained, allowing sustained ATP production in the absence of oxygen and preserving basic cellular homeostasis. In response to ischemic or hypoxic signals, the NMR heart can actively reduce its contractile frequency and force, thereby lowering overall energy expenditure[10]. This adaptive response is consistent with the intrinsically low metabolic rate observed at the whole-organism level in NMRs and further contributes to myocardial energy preservation under oxygen-limited conditions[99].

1.3.3 Naked mole-rat hearts retain neonatal-like features

Recent work from our lab has demonstrated that NMR heart undergoes a unique form of adaptive metabolic reprogramming, exhibiting a neonatal-like metabolic pattern. The NMR heart relies on a liver-like glycogen metabolism rather than FAO typically seen in the hearts of adult mammals[93]. Importantly, accumulating evidence from multiple studies indicates that a transition from FAO to glycolysis is a critical determinant of CM proliferative capacity and regenerative potential[68]. Motivated by these findings, our lab further investigated whether additional neonatal-like features are retained in the NMR heart beyond metabolism.

Adult NMR CMs are smaller and rounder than those of adult mouse, and they lack the large size, tight packing, and highly ordered arrangement that are characteristic of mature mammalian CMs (unpublished data) (Fig. 7A). Although the majority of NMR CMs are mononuclear and polyploid (Fig. 7B), they still retain approximately 20% mononuclear diploid CMs (Fig. 7C), a trait more permissive with cell-cycle re-entry[100]. Notably, genomic analyses reveal that *Tnni3k* has undergone pseudogenization in NMR, and loss of *Tnni3k* function has previously been associated with an increased proportion of mononuclear diploid CMs[49, 101]. In addition, NMR heart simultaneously expresses both adult (*Tnni3*) and fetal (*Tnni1*) isoforms of sarcomeric proteins (Fig. 7D), whereas isoform switching typically occurs during cardiac

development in other mammals[102, 103]. Finally, the cardiac mitochondrial morphology is immature and more similar to neonatal cardiac mitochondria (Fig. 7E), which correlates with reduced oxidative capacity and greater reliance on anaerobic metabolism, both of which are believed to be critical for promoting proliferation[74]. Taken together, the characterization of NMR myocardium across a panel of features revealed retention of neoteny, which is compatible with proliferation and regeneration.

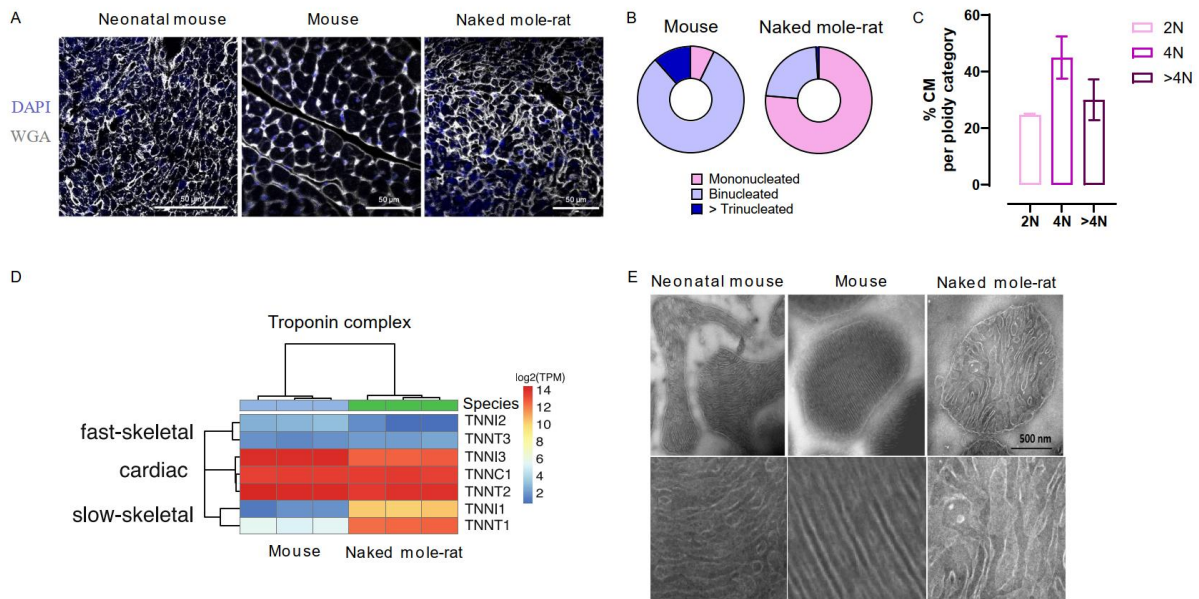


Fig. 7. Adult naked mole-rat myocardium retains neonatal-like structural and cellular features.

(A) Adult naked mole-rat cardiomyocytes resemble neonatal (P1) mouse cardiomyocytes in shape, size, and alignment, rather than those of adult mice (DAPI marks nuclei; WGA outlines cell borders). High-magnification images were taken from anatomically matched regions of the left ventricle in neonatal mouse, adult mouse, and naked mole-rat hearts. (B) Comparison of mono-, bi-, and multinucleated cardiomyocytes in mouse and naked mole-rat hearts reveals a higher proportion of binucleated cells in naked mole-rats, which may be more permissive to cell-cycle re-entry. (C) Ploidy number of mononucleated cardiomyocytes in the naked mole-rat. 20% of mononucleated cardiomyocytes in naked mole-rats are diploid representing a population most permissive for cell cycle re-entry. (D) Naked mole-rats do not undergo the typical troponin isoform switch seen in other mammals, retaining both neonatal skeletal and adult cardiac isoforms. (E) Naked mole-rat heart mitochondria exhibit immature morphology similar to neonatal mouse hearts, unlike the dense, organized cristae seen in adult hearts. Scale bar = 500nm.

1.3.4 Naked mole-rat hearts retain regenerative potential

As neonatal mammalian hearts retain regenerative capacity, we observed retention of neonatal features in adult NMR hearts. To further evaluate their ability to regenerate

lost myocardium, our lab performed left anterior descending (LAD) coronary artery ligation. At 7 days post-injury (dpi), NMR hearts exhibited pronounced scar expansion and tissue loss in the injured zone, accompanied by a reduction in ejection fraction, indicating impaired cardiac function (Fig. 8A). Although tissue loss was observed in the border zone, cells in this region displayed proliferative activity, which was not detected in the remote zone at this time point (Fig. 8B). In addition, co-staining for EdU and the CM marker Tnnt2 demonstrated CM proliferation in the border zone at 28 dpi (Fig. 8C). Notably, by 28 days and up to 6 months after injury, the injured area in NMR hearts was reduced, with fresh tissue replacing the damaged region and cardiac function gradually recovering (Fig. 8A and D). In contrast, in mice, the injured area remained unchanged over time, cardiac function did not recover, and extensive fibrosis was observed (Fig. 8A and D). Taken together, these results indicate that NMR hearts possess a regenerative capacity and recover cardiac function.

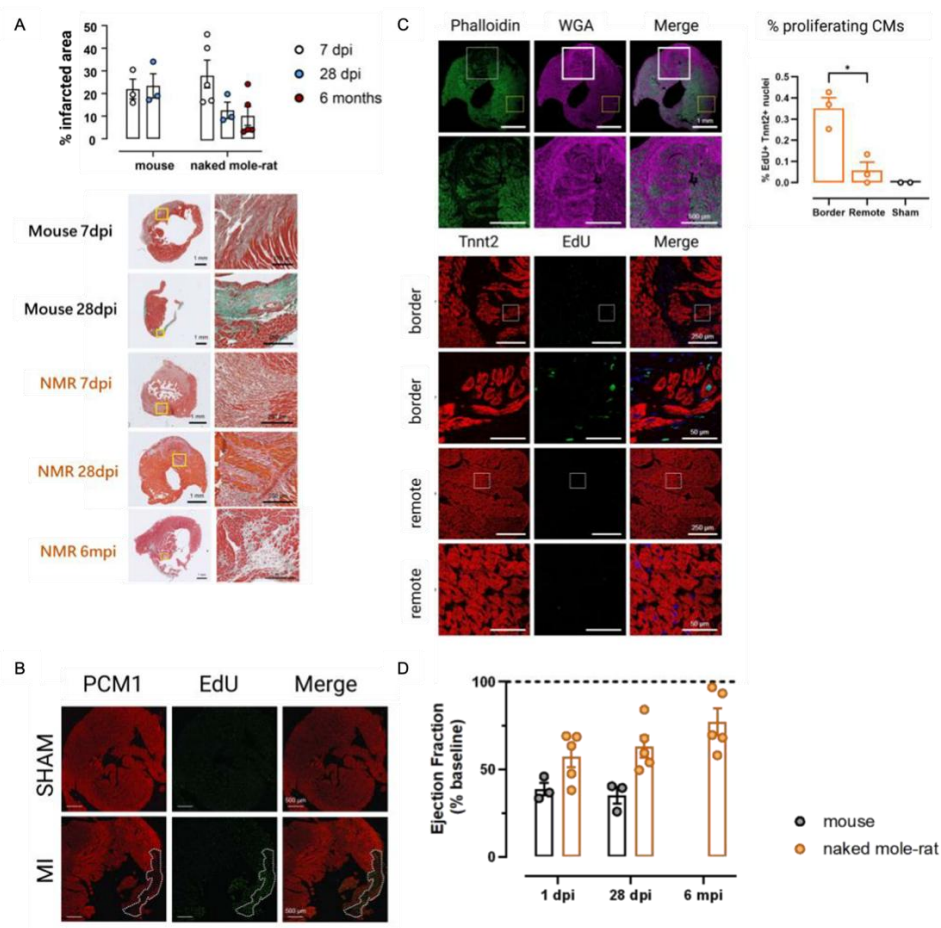


Fig. 8. Adult naked mole-rat (NMR) hearts after injury shows scar clearance and functional recovery.

(A) Quantification of infarcted region at 7 and 28 days post-injury (dpi) in mouse and NMR and 6 months post-injury in NMR. (B) Tissue section of heart at 7 dpi from SHAM and myocardial infarction (MI) NMR. PCM1 (red) stains cardiomyocytes, EdU (green) stains proliferating cells. (C) Tissue section from MI NMR heart at 28 dpi. The top panel shows a low-magnification image stained with phalloidin (live tissue) and WGA (total tissue), highlighting the border zone (white box) and remote zone (yellow box). The bottom panels show higher-magnification images of the border and remote zones at two different magnifications, stained for the cardiomyocyte marker TNNT2 (red) and the proliferation marker EdU (green). Quantification of proliferating cardiomyocytes in the border zone, remote zone, and the corresponding regions in sham-operated mice is shown on the right. (D) Ejection fraction, expressed as a percentage of baseline, at 7 and 28 dpi in mouse (black) and NMR (orange), and at 6 months post-injury in NMR only.

1.3.5 Implications of naked mole-rat cardiac biology for human heart disease

According to statistics from the World Health Organization, heart disease remains one of the leading causes of death globally[104]. This high mortality is largely attributable to the almost non-regenerative nature of adult human CMs, meaning that damage to heart muscle tissue, once incurred, is often permanent and irreversible. In contrast, certain vertebrate species, such as zebrafish, axolotl, and newts, have retained robust CM proliferation and regenerative abilities throughout life[38, 44]. Most mammals, however, progressively lose this capacity shortly after birth[105].

NMRs represent an exceptional case among mammals. As we mentioned before, they are extraordinarily long-lived and display remarkable tolerance to hypoxia as well as resistance to cardiac ischemia[10]. The natural habitat of NMRs - characterized by low oxygen and elevated carbon dioxide levels - closely resembles human pathological states such as ischemic heart disease, myocardial infarction, and chronic obstructive pulmonary disease[99]. Remarkably, despite these extreme environmental challenges, NMRs maintain stable cardiac function and exhibit only limited tissue damage, suggesting intrinsic protective and adaptive mechanisms that safeguard their hearts under stress. Their cardiac function does not decline with age[22]. Remarkably, our findings reveal that the NMR heart exhibits regenerative capacity. These unique features make the NMR a valuable model for human cardiovascular research. Exploring and elucidating the genetic and regulatory mechanisms underlying these adaptations could yield insights into the prevention and treatment of human heart diseases.

1.4 Chromatin architecture and epigenetic regulation

In multicellular organisms, gene expression is precisely regulated in both temporal and spatial dimensions during development, ensuring normal physiological functions. Chromatin plays a key role in this dynamic regulation by altering its physical structure and epigenetic modifications. Therefore, understanding how gene expression is regulated is essential for uncovering the mechanisms underlying development, environmental adaptation, and disease.

1.4.1 Chromatin

The fundamental unit of chromatin is the nucleosome, which consists of approximately 147 bp of double-stranded DNA wrapped around a histone octamer. Nucleosomes are connected like beads on a string to form chromatin, which can be further compacted into chromosomes. The histone octamer is composed of two H2A-H2B dimers and a tetramer formed by two H3-H4 dimers. In certain contexts, canonical histones can be replaced by histone variants, such as H3.3 and H2A.Z[106]. These variants can alter the physical properties of nucleosomes and influence the recruitment of specific regulatory factors, thereby affecting signaling pathways. Histone variants play critical roles in gene expression, development, and disease processes[107].

Chromatin can be classified into euchromatin and heterochromatin based on its level of compaction. Euchromatin is relatively loosely packed, providing sufficient access for transcription factors, RNA polymerase, and other regulatory proteins, thereby allowing gene transcription to occur. In contrast, heterochromatin is more densely compacted, which restricts access of transcriptional machinery and maintains chromatin in a repressive state, preventing gene expression[108]. Heterochromatin can be further subdivided into constitutive and facultative heterochromatin. Constitutive heterochromatin, typically found at centromeres and telomeres, is enriched in repetitive DNA sequences and remains transcriptionally silent across all cell types. Facultative heterochromatin, by contrast, is more dynamic and can switch between repressed and active states depending on developmental stage, cell type, or environmental cues[108, 109].

The dynamic organization of chromatin provides an important mechanism for regulating gene expression. Nucleosome positioning, histone composition, and chromatin compaction determine whether regulatory DNA sequences, such as

promoters and enhancers, are accessible to transcriptional regulators[110, 111]. When chromatin is open, TFs can recognize and bind their DNA motifs, recruit co-activators or chromatin-modifying enzymes, and promote transcriptional activation[112, 113]. Conversely, when chromatin is compacted, TF binding is restricted, limiting gene activation even when the relevant TFs are present. Therefore, chromatin structure does not simply package DNA, but actively controls how genetic information is interpreted.

1.4.2 Histone modification

Core histones, especially the N-terminal tails, contain numerous amino acid residues that are subject to post-translational modifications (Fig. 9). Major types of histone modifications include acetylation, methylation, phosphorylation, ubiquitination, and so on[114]. Depending on the degree of methylation, histone lysine residues can be mono-, di-, or tri-methylated, adding further complexity to the histone modification landscape. These modifications can alter the chromatin structure by changing its compaction and accessibility, thereby regulating gene expression. As one of the most crucial epigenetic mechanisms in eukaryotes, histone modifications play essential roles in controlling various physiological processes and developmental programs.

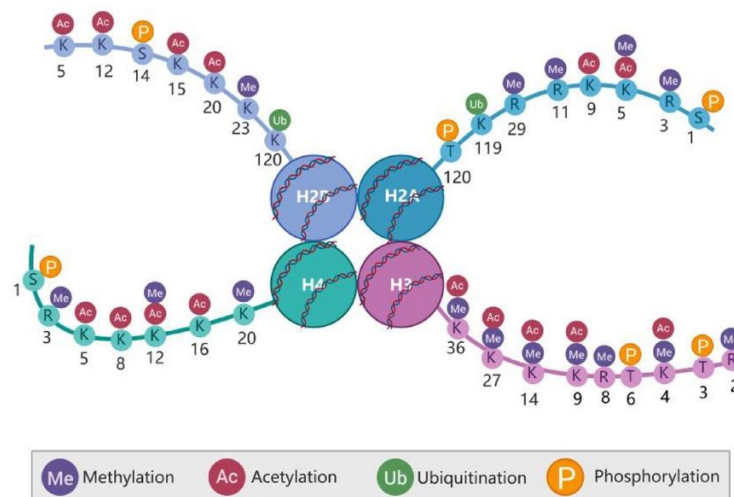


Fig. 9 Post-translational modifications of histone tails.

Nucleosomes, composed of two copies each of histones H2A, H2B, H3, and H4, contain flexible amino-terminal tails that are subject to extensive post translational modifications. These modifications regulate chromatin compaction and play critical roles in controlling gene expression programs.

Histone modifications can occur at various sites and are highly dynamic and reversible. This dynamic regulation is mediated by three classes of proteins commonly referred to as “writers”, “erasers” and “readers”[115]. “Writers” are enzymes that catalyze the addition of chemical groups to specific amino acid residues on histone tails, thereby establishing epigenetic marks, examples include histone methyltransferases (HMTs) and histone acetyltransferases (HATs). These marks can be removed by “erasers” such as histone demethylases (HDMs) and histone deacetylases (HDACs). “Readers” are proteins that contain specialized domains capable of recognizing and binding to specific histone marks, thereby mediating downstream biological functions[116]. For instance, the double-bromo domain of TAF1 recognizes acetylated histones and chromo domain of HP1 specifically bind to methylated lysines[117]. Thus, they work together to determine the patterns, levels, and functional outcomes of histone modifications.

The diversity of post-translational modifications and the numerous modifiable amino acid residues on histones allow for the formation of a wide range of epigenetic marks. Among them, methylation and acetylation are the most extensively studied. Methylation can either activate or repress gene expression, depending on the specific residue and the degree of methylation, whereas acetylation is generally associated with gene activation[114]. For instance, the activating methylation mark H3K4me3 recruits the TFIID complex to promote transcription initiation and is typically enriched at promoter regions[118]. H3K4me1 is often found at enhancer regions. In contrast, repressive methylation marks such as H3K27me3, catalyzed by the Polycomb repressive complex 2 (PRC2), are associated with facultative heterochromatin and gene silencing[119]. H3K9me3, on the other hand, is associated with constitutive heterochromatin, contributing to stable, long-term transcriptional repression[120]. The acetylation mark H3K27ac is commonly regarded as an indicator of transcriptional activity, and its enrichment at promoters and enhancers is often correlated with active gene expression.

1.4.3 Bivalent chromatin

Different combinations of histone modifications can lead to specific regulatory functions. For example, H3K4me3 together with H3K27ac usually marks active promoters, while H3K4me1 and H3K27ac often mark active enhancers[121]. In addition to these active elements, the co-existence of H3K4me3 and H3K27me3 defines bivalent promoters. These regions keep genes silent but poised for activation, especially those involved in development[122]. Similarly, enhancers marked by H3K4me1 and H3K27me3 are considered to be in a "poised" state, ready to become active upon receiving proper signals[123].

Bivalent domains were first discovered in mouse embryonic stem cells. Researchers observed this chromatin state in highly conserved noncoding elements. In these cells, developmental genes often carry both marks, allowing for rapid transcriptional activation during differentiation. Then, in differentiated cells, H3K27me3 is removed from specific developmental genes to allow their activation, while it remains on genes that are not needed for that cell type, keeping them repressed[122]. Their presence has been confirmed not only in mouse embryonic stem cells but also in human embryonic stem cells and in various differentiated tissues, such as the heart, liver, and brain[124]. This indicates that bivalency is a widespread chromatin feature, not limited to early development. These domains are also frequently found at CpG islands. However, all CpG sites marked by H3K27me3 are unmethylated[125].

Functionally, bivalent domains provide developmental flexibility by keeping key genes transcriptionally repressed but primed for rapid activation. In the hematopoietic system, H3K4 methylation plays a critical role in the maturation of blood cells. Loss of H3K4 methylation leads to a depletion of mature blood cell populations. At many affected promoters, the reduction of H3K4me3 is accompanied by an increase in H3K27me3, suggesting a shift toward a more repressive chromatin state that is associated with altered gene expression. Notably, essential hematopoietic regulators such as Klf4 and Ebf1 maintain bivalent chromatin marks in myeloid progenitor cells. These genes are selectively activated in specific downstream lineages, such as myeloid or lymphoid cells, during differentiation[126]. Furthermore, in cells with impaired H3K4 methylation, co-inhibition of H3K27 methylation can partially restore expression of certain target genes and alleviate associated phenotypic defects[127]. This highlights the critical role of bivalent chromatin in maintaining normal hematopoietic function and regulating the proper differentiation and maturation of blood cells. In addition, during liver

regeneration, bivalent chromatin can lose the repressive H3K27me3 mark, allowing bivalent genes to be activated[128]. These genes promote regeneration and are mainly involved in cell proliferation and differentiation, development and fate determination, as well as DNA replication, repair, and recombination[129].

1.4.4 Chromatin and epigenetic regulation in heart regeneration

During mammalian heart development, the dynamic regulation of gene expression is critically orchestrated by chromatin remodeling and epigenetic modifications, such as histone acetylation and methylation, as well as changes in chromatin accessibility[130]. These epigenetic mechanisms shape the transcriptional programs necessary for heart maturation, including the silencing of fetal gene networks and the activation of adult-specific cardiac genes[131, 132]. Therefore, understanding the epigenetic mechanisms underlying heart maturation may provide strategic insights into promoting heart regeneration.

Recent studies have demonstrated that metabolic intermediates influence epigenetic landscapes during postnatal heart development. For instance, α -Ketoglutarate (α -KG), a key TCA cycle metabolite, has been identified as one of the most significantly different metabolites between mouse hearts on postnatal day 0.5 and day 7. Injection of α -KG into adult mice with myocardial infarction has been shown to promote cardiac regeneration by modulating histone methylation patterns. Specifically, α -KG enhances the activity of histone demethylases, leading to a reduction in H3K27me3 enrichment at promoters of cell cycle genes and a decrease in H3K4me3 marks at promoters of maturation-associated genes[133]. This indicates that metabolic-epigenetic crosstalk can reactivate the proliferative program in adult CMs. Epigenetic mechanisms also control the silencing of fetal-specific genes during maturation. For example, *Tnni1* silencing is associated with the loss of active histone marks such as H3K27ac, H3K4me3, H3K9ac, and H3K36me3, highlighting the role of histone modifications in the developmental transition of cardiac gene expression programs[134]. In addition, deletion of histone methyltransferases such as *Ezh2*, a key component of the PRC2, leads to increased expression of fetal genes and partial reactivation of CM proliferation in adult hearts[135]. These findings suggest that the balance between active and repressive chromatin states contributes to the maintenance of adult CM identity and the restriction of regenerative capacity.

Other studies further support the idea that adult mammalian CMs retain latent regenerative potential that can be influenced by chromatin remodeling. Activation of pro-regenerative pathways, such as YAP signaling, has been shown to partially reprogram chromatin accessibility in adult CMs and to induce fetal or proliferative gene programs[75, 136]. Inhibition of cell-cycle suppressive regulators such as DYRK1A has also been associated with increased active histone marks, including H3K4me3 and H3K27ac, at the promoters of proliferation-related genes, thereby promoting CM cell-cycle activity and cardiac repair[137]. Moreover, regenerative models such as zebrafish exhibit dynamic injury-responsive enhancer activation during heart regeneration, further highlighting the importance of chromatin plasticity in enabling repair-associated gene expression[138].

Collectively, these findings support a model in which chromatin and epigenetic regulation not only facilitate CM maturation during development but also represent modifiable barriers to heart regeneration. Targeting these mechanisms may thus offer therapeutic strategies to promote myocardial repair in adult hearts.

2 Aim of the thesis

Previous studies from our lab and others have shown that naked mole-rats exhibit unique phenotypic traits distinct from those of other mammals, including remarkable hypoxia tolerance, a neonatal-like metabolic program, and enhanced heart regenerative capacity[2, 10, 93] (Fig. 6-8). Given that neonatal programs and metabolic states have been implicated in sustaining and driving cardiac regeneration[41, 68], I hypothesize that distinctive metabolic and neonatal-like programs observed in NMRs hearts contribute to both hypoxia resistance and regenerative capacity. However, the molecular mechanisms underlying these programs remain largely unknown. Therefore, the aim of this thesis is to investigate the genetic and epigenetic architecture underlying these programs.

Specifically, this study aims to address the following questions:

1. Do adult naked mole-rat hearts undergo incomplete postnatal maturation at the transcriptional and epigenetic levels?
2. How are the adaptive evolutionary phenotypes of adult naked mole-rat hearts regulated, and which transcription factors play central roles in this regulation?
3. Under hypoxic conditions, which genes and pathways are rapidly induced to support physiological stability and stress resilience in naked mole-rat hearts?

3 Methods and materials

3.1 Cardiomyocyte nuclei isolation and immunoprecipitation

Adult and P1 hearts were excised, rinsed in ice-cold PBS buffer, and cut into small pieces. Unless otherwise stated, all subsequent procedures were performed on ice or at 4 °C using pre-chilled buffers and low-binding tubes. Tissue fragments were transferred to gentleMACS C Tubes (130-093-237, Miltenyi Biotec) containing 2 ml of Nuclei Extraction Buffer (130-128-024, Miltenyi Biotec). Tissue dissociation was performed using a gentleMACS Dissociator program optimized for heart nuclei isolation. Following dissociation, samples were incubated on ice for 5 min to facilitate nuclei release. The resulting nuclei suspension was filtered through a 70 µm cell strainer, and the strainer was rinsed with an additional 2 ml of Nuclei Extraction Buffer. Nuclei were then pelleted by centrifugation at 300 × g for 5 min at 4 °C. The nuclei pellet was resuspended in Washing Buffer (WB), consisting of 20 mM Tricine (pH 7.8), 250 mM sucrose, 25 mM KCl, 5 mM MgCl₂, 1 mM DTT, 0.15 mM spermine, 0.5 mM spermidine, cOmplete™ EDTA-free Protease Inhibitor Cocktail (05892791001, Roche), and 0.4% IGEPAL CA-630. The suspension was filtered again through a 30 µm strainer, centrifuged under the same conditions, and resuspended in WB.

For cardiomyocyte nuclei enrichment, Dynabeads Protein G (10003D, Invitrogen) were washed twice with WB and resuspended to the original bead volume. To reduce non-specific binding, a fraction of the washed beads was added to the nuclei suspension and incubated for 15 min at 4 °C with gentle rotation. Beads were then removed magnetically and the pre-cleared supernatant was transferred to a new tube containing anti-PCM1 antibody (HPA023370, Sigma) diluted 1:200 in WB. After 45 min incubation at 4 °C with gentle rotation, the remaining pre-washed Dynabeads were added and incubated for an additional 25 min. Beads were collected using a magnetic stand and washed once with fresh Washing Buffer, passed through a 20 µm strainer, and washed an additional seven times with WB.

3.2 ATAC-seq library preparation

A total of 100,000 isolated PCM1-positive nuclei were resuspended in transposition mix [1× TD buffer and TDE1 enzyme (20034197, Illumina Nextera DNA Tagmentation

Kit; enzyme diluted 1:20), 0.01% digitonin, 0.1% Tween-20, PBS]. The transposition reaction was carried out for 30 min at 37 °C.

Following transposition, DNA was purified using the DNA Clean & Concentrator-5 kit (D4013, Zymo Research) and eluted in molecular biology grade water. Libraries were amplified using NEBNext High-Fidelity 2× PCR Master Mix (M0541S, New England Biolabs) under the following conditions: 72 °C for 5 min, 98 °C for 30 s, followed by N cycles of 98 °C for 10 s, 63 °C for 30 s, and 72 °C for 1 min.

The optimal number of PCR cycles was determined by qPCR, stopping amplification at one-third of the plateau phase. Final libraries were purified using AMPure XP beads (A63880, Beckman Coulter) and eluted in 10 mM Tris-HCl (pH 8.0). Library quality and concentration were assessed using Qubit and TapeStation. Sequencing was performed on a NovaSeq 6000 platform.

PF: 5'-AATGATACGGCGACCACCGAGATCTACAC [i5 index]
CTATTCGTCTCGGCAGCGTCAGATGTG-3'

PR: 5'- CAAGCAGAAGACGGCATACGAGAT [i7 index]
GTCTCGTGGGCTCGGAGATGT-3'

3.3 RNA-seq library preparation

Adult and P1 hearts were excised, rinsed in ice-cold PBS, and cut into small pieces. Tissue fragments were transferred to Precellys tubes containing 1 mm zirconia beads (N038.1, Carl Roth), and 1 ml of TRI Reagent was added. Samples were homogenized using a Precellys homogenizer with two cycles at 6500 rpm for 30 s, separated by a 15 s pause, followed by incubation at room temperature for 5 min. The lysates were transferred to new microcentrifuge tubes, and chloroform was added. Samples were vigorously shaken, incubated briefly at room temperature, and centrifuged at 4 °C. The upper aqueous phase was transferred to a new tube and mixed with an equal volume of 96% ethanol. RNA was further purified using the SV Total RNA Isolation System (Z3105, Promega) according to the manufacturer's instructions. Briefly, the mixture was loaded onto a spin column, centrifuged, and washed. On-column DNase I digestion was performed for 15 min at room temperature. The column was then washed with DNase Stop Solution followed by two washes with RNA Wash Solution. RNA was eluted in nuclease-free water and stored at -80 °C. RNA-seq libraries were

prepared by an external service provider. Sequencing was performed on a NovaSeq X platform.

3.4 RNA-Seq Data Analysis

Raw RNA-seq reads were first processed to remove adapter contamination and low-quality sequences using Trim_Galore (v0.6.6) via calling the Cutadapt tool. After quality filtering, cleaned reads from each species were quantified using Salmon (v1.5.1). For each species-specific transcriptome reference, the longest transcript of each gene was selected as the representative transcript and used to build the Salmon index. The representative transcript was identified using a custom Python script. Briefly, when different isoforms of the same gene had different coding sequence (CDS) lengths, the isoform with the longest CDS was selected. If multiple isoforms had identical CDS lengths, the isoform with the longest transcript length was retained. The same strategy was used to extract the longest protein sequence for each gene, which was subsequently used for ortholog identification. After Salmon quantification, transcript-level TPM values, read counts, and effective transcript lengths were collected for each sample.

To enable cross-species comparison between mouse and naked mole-rat, orthologous genes were identified based on reciprocal BLAST searches. The longest protein sequences from mouse (GRCm39, Ensembl release104) and naked mole-rat (Heterocephalus_glaber_female, Ensembl release110) were aligned against each other using blastp (BLAST+ v2.10.1), with parameters of “-evalue 1e-05; -max_target_seqs 1”. Gene pairs were defined as orthologous when the forward and reciprocal BLAST searches identified each other as the best hit. Based on the resulting orthologous gene relationships, Salmon quantification outputs from mouse and naked mole-rat were integrated into cross-species transcript-by-sample matrices. The resulting count tables were used for downstream differential expression and comparative transcriptomic analyses.

Differential expression analysis was performed using Salmon-derived transcript counts. For each tissue-specific count matrix, lowly expressed genes were removed by excluding genes with a total read count below 10 across all samples. The remaining count matrix was analyzed using DESeq2 (v1.42.1) package, with normalization

performed using median of ratios method. Because orthologous transcripts may differ in length between mouse and naked mole-rat, we incorporated an additional transcript length correction into the DESeq2 workflow to minimize potential bias in cross-species expression comparisons. Specifically, the Salmon-estimated effective transcript length matrix was supplied to the DESeq2 'DESeqDataSet' object, allowing effective length information to be included during normalization and downstream statistical testing. Gene relative expression heatmaps were generated using row-scaled Z-scores of log₂-transformed DESeq2-normalized counts, whereas box plots were generated using log₂-transformed DESeq2-normalized counts.

For cross-species comparisons of adult samples and comparisons across developmental stages, genes were considered significantly differentially expressed if they met the following criteria: adjusted p-value < 0.01, absolute log₂ fold change > 1.5, and maximum TPM across all samples > 1. For differential oxygen conditions, genes were defined as significantly differentially expressed using adjusted p-value < 0.05, absolute log₂ fold change > 1, and maximum TPM across all samples > 1. Differential expression was modeled using a negative binomial generalized linear model, and statistical significance was assessed using the Wald test. P-values were adjusted for multiple testing using the Benjamini-Hochberg method to control the false discovery rate (FDR).

3.5 Principal Component Analysis

Principal component analysis (PCA) was performed to assess global transcriptional differences among samples based on gene expression profiles. Gene-level expression matrices were imported into R (v4.3.3) for analysis. Prior to PCA, genes with zero variance across all samples were removed to avoid numerical instability and to ensure that only informative genes contributed to the analysis.

The filtered expression matrix was transposed so that samples were treated as observations and genes as variables. PCA was conducted using the `prcomp` function in R with scaling enabled (`scale. = TRUE`), allowing each gene to contribute equally to the analysis. Principal component scores were extracted, and the proportion of variance explained by each principal component was calculated from the PCA output. PCA visualizations were generated using the `ggplot2` package (v3.5.3).

3.6 ChIP-seq data analysis

Read mapping and filtering. Low-quality reads and adapter were filtered using Trim Galore (v0.6.10) (www.bioinformatics.babraham.ac.uk/projects/trim_galore). The NMR and mouse genome, which has been publicly available on Ensembl database release110 (Naked_mole-rat_maternal and GRCm39), were used as the reference genomes for analyses. The cleaned reads were then aligned to the reference genomes using bowtie2 (v2.2.9) under default parameters. Then the un-mapped and multi-mapping reads were filtered out (-q 30) using SAMtools (v1.13). After alignment, reads on chromosome mitochondrial were removed using SAMtools. Then PCR duplicates were removed using MarkDuplicates function in Picard (v2.1.1) with "REMOVE_DUPLICATES=true".

To facilitate genome browser-based visualization, we generated normalized signal tracks from the final ChIP-seq alignment files using deepTools (v3.5.2), then converted into normalized bigWig format using the bamCoverage function. The following parameters were used: --binSize 10 --normalizeUsing RPKM. The resulting bigwig files represent genome-wide histone modification signal intensities and were used for visual inspection and qualitative comparison in IGV (v2.11.0; Integrative Genomics Viewer).

Peak calling. For the analysis of chromatin histone marks, to avoid artificial differences in signal strength due to differences in sequencing depth, all the data sets were adjusted to a maximum depth of 50 million reads (the median reads depth over all consolidated data sets) before peak calling. For narrow peaks, we performed peak calling for H3K27ac, H3K4me1, and H3K4me3 ChIP-seq datasets using MACS2 (v2.2.8) with "--keep-dup all --qvalue 0.05". For H3K27me3, peaks were called using epic2 (v0.0.26), a tool specifically designed for identifying broad domains of enrichment. Input controls were used to model the background signal distribution. Following individual peak calling, we defined consensus peaks for each histone modification to ensure reproducibility and biological robustness. Specifically, peaks were retained only if they were present in at least two biological replicates of the same histone mark.

Remove greylist from peaks. To ensure the robustness and reliability of our peak data analysis, we identified greylist in input ChIP experiments, which with unusually high signal intensities, indicative of potential non-specific binding or other artifacts. These

regions were identified using the GreyListChIP (<https://bioconductor.org/packages/release/bioc/html/GreyListChIP.html>) tool, based on the input samples corresponding to each histone mark. Final peaks were filtered by subtracting greylist regions using BEDTools (v2.31.1).

To annotate functional regulatory elements in the genome, we classified histone modification-enriched regions into promoters and enhancers based on combinatorial chromatin signatures. Active promoters were defined as regions where H3K27ac peaks overlapped with H3K4me3 peaks, representing promoter regions marked by both activation and transcription initiation marks. Active enhancers were defined as H3K27ac peaks overlapping with H3K4me1 peaks. Potential enhancers were defined as H3K27ac peaks not overlapping with either H3K4me3 or H3K4me1, representing putative enhancer elements lacking canonical promoter or enhancer marks. All overlaps and set operations were performed using BEDTools. Histone mark-specific peaks used in this analysis were pre-processed to remove greylist regions as described above.

3.7 ATAC-seq data analysis

Paired-end ATAC-seq raw reads underwent initial quality control using FastQC (v0.12.1; www.bioinformatics.babraham.ac.uk/projects/fastqc). Adapter sequences and low-quality bases (-q 30) were trimmed using Trim Galore (v0.6.10), which internally uses Cutadapt (v4.4). Trimmed reads were aligned to the mouse reference genome mm39 or naked mole-rat reference genome Naked_mole-rat_maternal (Ensembl database release110) assembly using bowtie2 (v2.5.4) with default parameters. After alignment, mitochondrial reads were removed using SAMtools (v1.19.2), as they are overrepresented in ATAC-seq and can confound signal interpretation. PCR duplicates were subsequently marked and removed using Picard (MarkDuplicates). The resulting high-quality BAM files were used for subsequent analyses.

To identify regions of accessible chromatin, we used MACS2 (v2.1.2) for peak calling. BAM files were first converted to BED format, and MACS2 was run with the following parameters: -f BED --nomodel --shift -100 --extsize 200 --keep-dup all -q 0.05. Coverage tracks requiring bigwig files and heatmaps were generated using deepTools

(v3.5.2). For downstream analysis, we used peaks only present in at least two biological replicates.

3.8 Orthologous regions identification

To identify orthologous genomic regions between *Mus musculus* and *Heterocephalus glaber*, we performed pairwise whole-genome alignment using the LAST alignment suite (v1389). The *Mus musculus* genome (mm39) was used as the reference.

First, the mouse genome was indexed to construct a reference alignment database using lastdb with the following parameters: lastdb -uNEAR -cR11. A substitution scoring matrix was then trained with last-train using both the reference (mouse) and query (NMR) genomes to optimize alignment parameters based on the specific evolutionary divergence between the two species.

Pairwise alignment was conducted with the trained scoring matrix using lastal, followed by post-processing with a split-alignment algorithm to extract optimal local alignments and eliminate ambiguous matches. To retain high-confidence reciprocal best alignments, we applied maf-swap and last-split recursively and sorted the output.

The resulting alignment in MAF (Multiple Alignment Format) was converted into PSL format, and subsequently into chain format using axtChain, allowing the representation of alignment blocks with gap penalties optimized for large-scale genomic rearrangements. Both genomes were first converted to 2bit format for compatibility with chain file generation using faToTwoBit. This produced chain files in the direction of mouse (reference) to NMR (query). However, to allow coordinate conversion from NMR to mouse, we performed a chain file inversion step using swapchain.

Finally, individual chain files were concatenated into a unified whole-genome chain file representing the alignment between mouse and NMR genomes. The chain files were later used for coordinate conversion across species using LiftOver (http://hgdownload.soe.ucsc.edu/admin/exe/linux.x86_64/liftOver), enabling comparative analysis of regulatory elements in a shared genomic coordinate framework.

3.9 Differential active peaks identification

To identify differentially active regulatory regions between *Mus musculus* and *Heterocephalus glaber*, we focused on H3K27ac consensus peaks. We first established a set of orthologous regions corresponding to H3K27ac-enriched loci. Specifically, H3K27ac consensus peaks identified in the NMR were first mapped to the mouse genome using the LiftOver tool (-minMatch=0.3), applying the previously generated swapped chain file. This allowed the projection of NMR regulatory regions onto the mouse genomic coordinate system. The lifted-over regions were then merged with the H3K27ac consensus peaks originally identified in mouse using BEDTools, resulting in a unified set of putative orthologous regulatory elements in mouse coordinates. Peaks that did not overlap between species were considered non-orthologous, representing species-specific active regulatory elements.

To ensure reciprocal alignment and obtain orthologous coordinates in both species, the merged peak set was subsequently lifted back to the NMR genome using the original (non-swapped) chain file. Only successfully mapped regions were retained as orthologous peak pairs. This two-step LiftOver strategy allowed us to define a high-confidence set of orthologous H3K27ac peaks shared between mouse and NMR, which was then used as the basis for quantitative comparison of peak signal intensity and identification of differentially active peaks between the two species.

After defining orthologous H3K27ac peak regions between mouse and NMR, we quantified the H3K27ac signal intensity within these regions across all biological replicates to enable cross-species comparison of chromatin activity. For each biological replicate, final BAM files were used to extract raw read counts within orthologous peak regions using BEDTools, with corresponding input control read counts subtracted to account for background signal.

The resulting raw count matrix, comprising counts from all samples and peaks, was then analyzed using DESeq2, which internally performed normalization to correct for differences in library size and sequencing depth via size factor estimation. DESeq2 employed a negative binomial generalized linear model to estimate variance and test for differential peak enrichment between the two species. Peaks with statistically significant differences (adjusted p-value < 0.01) and biologically meaningful fold changes ($|\log_2 \text{fold change}| \geq 1.5$) were identified as differentially active peaks.

3.10 Annotation of peaks to nearby genes

To assign biological context to the identified differential peaks and non-orthologous peaks, we performed genomic annotation of peaks to nearby genes using the ChIPseeker (v1.38.0) package in R.

Peak annotation was conducted for each group of peaks. The function `annotatePeak` was used to annotate peaks relative to transcription start sites (TSSs) based on the `Mus_musculus.GRCm39.110.chr.gff3` annotation database for mouse and `Heterocephalus_glaber_female.Naked_mole-rat_maternal.110.chr.gff3` for NMR. Peaks were categorized according to their genomic features, including promoter (typically defined as ± 2 kb around TSS), 5'UTR, 3'UTR, exon, intron, and intergenic regions.

Additionally, each peak was assigned to the nearest gene to facilitate downstream analyses such as functional enrichment. The distribution of peak annotations across genomic features was summarized and visualized using `plotAnnoPie`. This approach enabled the linking of putative regulatory elements with their associated target genes, then with these genes and DEGs together to identify differentially regulated genes (DRGs).

3.11 Conservation score

To further validate the reliability of the identified orthologous regulatory elements, we hypothesized that orthologous sequences should exhibit higher evolutionary conservation compared to non-orthologous sequences. We obtained the phastCons 60-way vertebrate conservation scores in the form of bigWig files mapped to the *Mus musculus* mm10 reference genome from the UCSC Genome Browser (<http://hgdownload.soe.ucsc.edu/goldenPath/mm10/phastCons60way/mm10.60way.phastCons.bw>). Coordinates of both orthologous and non-orthologous H3K27ac peaks in mouse were converted to mm10 coordinates using LiftOver from `ucscTools` to ensure consistency with the conservation data.

For each peak set, conservation scores at all base positions were extracted from the phastCons bigWig files. The average conservation score across all bases within each

peak was calculated to represent the peak-level conservation score, ranging from 0 (least conserved) to 1 (highly conserved). To statistically compare conservation between orthologous and non-orthologous peaks, we performed a Wilcoxon rank-sum test in R (v4.3.3). The test revealed a highly significant difference, with a p-value less than $2.2e-16$, indicating that orthologous peaks are significantly more conserved than non-orthologous peaks.

To visualize these differences, boxplots were generated using the ggplot2 package (v3.5.3) in R, displaying the distribution of conservation scores for both orthologous and non-orthologous peak groups.

3.12 Gene functional enrichment analysis

Genes were subjected to Gene Ontology (GO) pathway enrichment analyses to investigate their biological functions and involved pathways. GO enrichment analysis was performed using clusterProfiler package (v4.10.1) with enrichGO function, focusing on the biological process ontology. To reduce redundancy among enriched GO terms, the simplify function was applied with a similarity cutoff of 0.7, retaining terms with the lowest adjusted p-values. Visualization of the top significantly enriched GO terms was performed using ggplot2 (v3.5.3).

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was using clusterProfiler package (v4.10.1) with enrichKEGG function. Significantly enriched pathways were identified based on a p-value < 0.05 (Fisher's exact test).

3.13 Transcription factor binding motif enrichment analysis

To investigate potential upstream regulators of species-specific regulatory activity, we performed transcription factor binding motif enrichment analysis on differential H3K27ac peaks. To further ensure that the identified regulatory events occurred within CMs and to refine the scope of regulatory elements, we intersected the more active NMR peaks with CM-accessible peaks (ATAC-seq) using bedtools. Based on prior functional annotations, these intersected peaks were categorized into NMR more active promoter and NMR more active enhancer sets. Similarly, the less active NMR peaks (i.e., more active in mouse) were intersected with mouse CM-accessible peaks

and divided into mouse more active promoter and mouse more active enhancer sets. Motif enrichment analysis for each of the four peak sets was then performed using HOMER (v4.11) with the *findMotifsGenome.pl* with parameters “-size given” and “-mset vertebratesmotif” discovery tool.

In addition, for each peak set, we generated a corresponding background set by randomly shuffling (using bedtools shuffle) H3K27ac peaks that did not show significant differences in activity between species. The background sets were constructed to match both the number and coverage of peaks in the foreground sets, and were provided as input to the *findMotifsGenome.pl* tool via the -bg parameter. This strategy ensured that motif enrichment was evaluated against an appropriate background, thereby yielding robust and biologically meaningful enrichment results.

3.14 Footprinting analysis

To assess transcription factor (TF) activity within accessible chromatin regions, we performed ATAC-seq-based motif footprinting using TOBIAS (v0.17.1). Species-specific genome assemblies were used for all steps. First, ATACCorrect was applied to bias-correct Tn5 transposase insertion signals, generating corrected bigWig signal tracks. Next, ScoreBigwig was used to calculate footprint scores across the genome, quantifying local decreases in accessibility indicative of protein-DNA binding. Position frequency matrices of vertebrate TF motifs were obtained from the JASPAR 2022 vertebrate core non-redundant database (https://jaspar.elixir.no/download/data/2022/CORE/JASPAR2022_CORE Vertebrates _non-redundant_pfm_meme.txt). Finally, BINDetect was used to scan the accessible regions for motif occurrences.

3.15 Nucleosome positions and occupancy

Nucleosome positioning and occupancy were inferred from ATAC-seq data using NucleoATAC (v0.3.4). For each sample, NucleoATAC was then run with the corrected BAM files and corresponding peak regions obtained from ATAC-seq peak calling process to generate genome-wide nucleosome occupancy profiles and nucleosome dyad position calls. Output files included bigWig tracks for nucleosome occupancy and BED files for predicted nucleosome positions, which were visualized in IGV.

3.16 Fast-evolving elements identification

To identify fast-evolving elements in the NMR, we first obtained 120-way mammalian conserved elements, multiple genome alignment (MAF) files for each chromosome and a corresponding neutral model from previous study (<https://bds.mpi-cbg.de/hillerlab/120MammalAlignment/Human120way/>)[139]. Fast-evolving conserved elements in the NMR lineage were then detected using phyloP from the PHAST package (v1.5) in CONACC mode with the likelihood ratio test (LRT) method. The final fast-evolving conserved elements was defined as those showing a statistically significant deviation from the neutral model (altsubscale > 1 and adjusted *P*-value < 0.05, Benjamini–Hochberg correction).

3.17 Gene Set Enrichment Analysis

GSEA was performed to identify pathways and biological processes significantly associated with the gene expression changes. Enrichment analysis was conducted in R using the clusterProfiler package (v4.10.1). All genes were ranked according to their log2 fold-change values, and Gene Set Enrichment Analysis (GSEA) was performed against gene sets obtained from the Molecular Signatures Database (MsigDB), including the Hallmark, KEGG, Reactome, and GO Biological Process collections, as well as several custom gene sets constructed based on our biological interests. Significantly enriched pathways and biological processes were defined as those with a false discovery rate (FDR) *q*-value < 0.05. Visualization of enrichment results was carried out using the gseaNb function from clusterProfiler.

3.18 The dN/dS analysis of ESRRG

15 mammalian species covered different orders were selected to perform dN/dS analysis of ESRRG. Coding sequences of *Esrrg* from each species were downloaded from the NCBI database. Branch-specific dN/dS ratios (ω) across the mammalian phylogeny were estimated using CODEML in the PAML (v4.10.1). We used the branch model with freely varying omega (model = 1, NsSites = 0) to infer dN/dS across all branches separately.

The species tree used for this analysis was specified as:

(((((SpermWhale,Pig),Horse),((Dog,Cat),MalayanPangolin)),(CommonVampireBat,BigBrownBat)),Hedgehog),(((Mouse,GoldenSpinyMouse),NakedMoleRat),Human)),(Elephant,Armadillo));

4 Results

4.1 Neonatal-like transcriptional and epigenetic landscapes in adult naked mole-rat hearts

4.1.1 Retention of neonatal-like transcriptional profile in adult naked mole-rat heart

Adult naked mole-rat (NMR) hearts retain several neonatal-like characteristics, including preserved neonatal cardiomyocytes (CMs) morphology and immature mitochondrial structure (Fig. 7). To examine whether these features are reflected at the transcriptional level, we obtained RNA-seq data from mouse and NMR hearts at P1 and adult stages (NMR: ~3 years old; mouse: ~3 months) (Table 1). Principal component analysis (PCA) revealed clear separation of samples by developmental stage and species (Fig. 10A). While adult NMR hearts were clearly distinct from neonatal hearts along the primary developmental axis (PC1, 42.83% of variance), they retained similarities with neonatal samples along PC3 (6.38% of variance) (Fig. 10B). Consistent with these global patterns, differential expression analysis revealed 992 upregulated and 1,032 downregulated genes between adult and P1 mouse hearts (Fig. 10C). In contrast, NMR showed substantially fewer changes during the same developmental transition, with 485 up-regulated and 644 down-regulated genes in adult compare to P1 (Fig. 10D). This reduction in the number of differentially expressed genes (DEGs) indicate a reduced degree of transcriptional remodeling during postnatal maturation in NMR hearts.

Table 1. Summary of RNA-seq and ATAC-seq data from P1 and adult in mouse and NMR CMs.

Data type	Conditions	Tissue	Replicates(NMR/MM)
RNA-seq	Adult	Heart	3/4
	P1	Heart	4/5
ATAC-seq	Adult	Cardiomyocyte	3/2
	P1	Cardiomyocyte	3/2

To quantify the similarity of developmental expression changes across species, we calculated Spearman correlation coefficients between the $\log_2$ fold changes of orthologous genes in mouse and NMR. A moderate positive correlation was observed ($r = 0.41$, $p < 2.2 \times 10^{-16}$), indicating conservation of neonatal-to-adult expression

trends, while also suggesting divergence between species (Fig. 10E). Consistent with this, quadrant analysis showed that a considerable proportion of genes (~35%) exhibit opposing expression changes between species during heart development (Fig. 10F). Furthermore, the top 300 genes upregulated in neonatal (P1) mouse hearts compared with adult mouse hearts were used to define a neonatal gene signature. We then performed GSEA on the adult NMR versus adult mouse heart dataset using this gene set, revealing significant enrichment of the neonatal signature in adult NMR hearts (Fig. 10G). Together, these findings demonstrate that adult NMR hearts retain a neonatal-like transcriptional signature despite postnatal maturation, supporting the idea that the NMR heart maintains an immature molecular state that may contribute to its remarkable stress resilience and regenerative capacity.

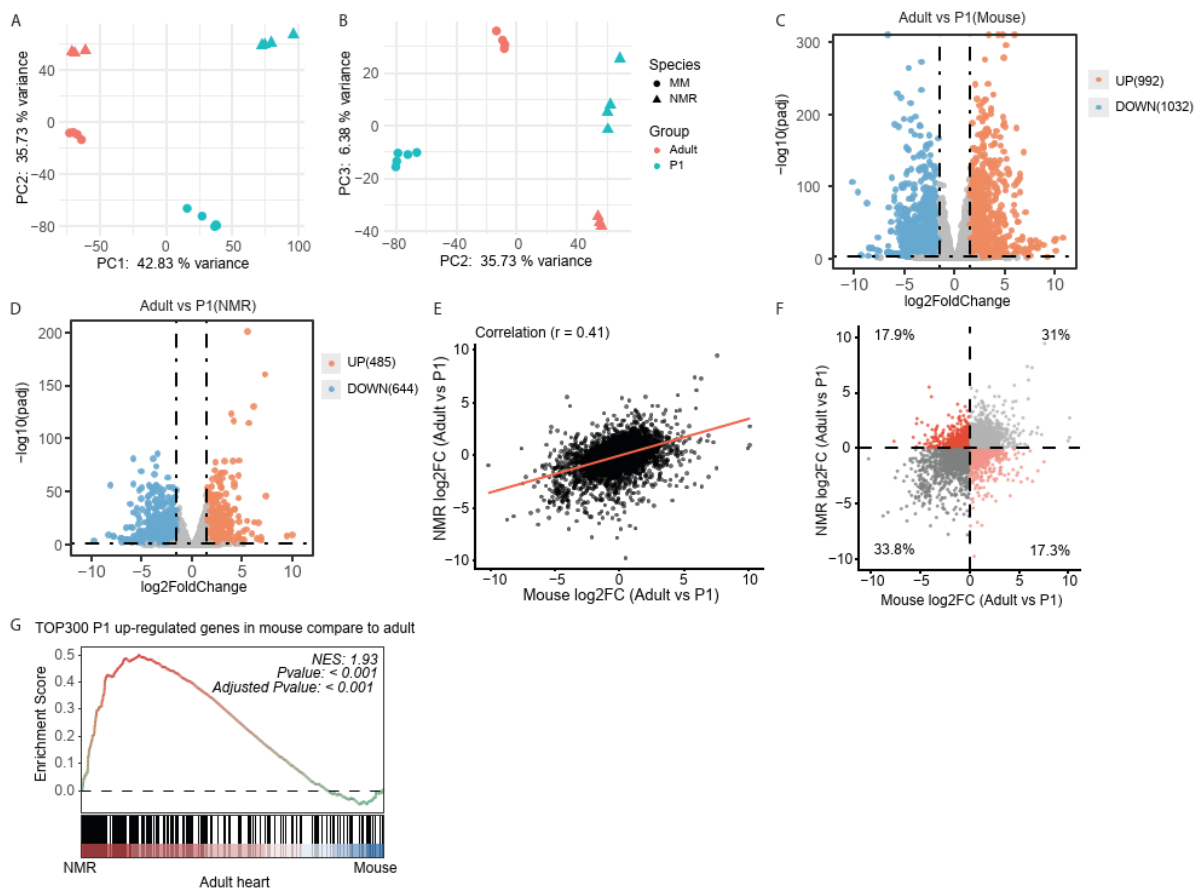


Fig. 10 Adult naked mole-rat (NMR) hearts retain a neonatal-like transcriptional state.

(A and B) Principal component analysis (PCA) of P1 and adult hearts. (A) PC1 versus PC2, where PC1 captures developmental stage differences, and PC2 separates samples by species. (B) PC2 versus PC3, showing that along PC3 - capturing neonatal-like variance - adult NMR hearts cluster closer to P1 samples, indicating preservation of neonatal transcriptional features. (C and D) Volcano plots showing differentially expressed genes between P1 and adult hearts in mouse (C) and NMR (D). Genes with

$|\log_2 FC| > 1.5$ and p -adjusted value < 0.01 are highlighted. (E) Scatter plot of developmental expression changes between species. Spearman correlation ($r = 0.41$, $p < 2.2 \times 10^{-16}$) indicates partial conservation of neonatal-to-adult expression trends. The red line indicates the linear regression fit. (F) Quadrant plot showing the relationship between developmental gene expression changes in mouse and NMR hearts. Each point represents an orthologous gene, with the x-axis indicating $\log_2$ fold change (Adult vs P1) in mouse and the y-axis indicating $\log_2$ fold change in NMR. Dashed lines indicate no change, dividing genes into four groups by directionality. The proportion of genes in each quadrant is indicated. Genes in the upper right (light grey) and lower left (grey) quadrants show conserved changes, whereas genes in the upper left (red) and lower right (light red) quadrants exhibit opposing changes between species. (G) GSEA showing that the top 300 genes upregulated in neonatal mouse hearts compared to adult are significantly enriched among genes highly expressed in adult NMR hearts compared to adult mouse hearts.

To explore whether the persistence of neonatal-like transcriptional states in adult NMR hearts is mirrored at the chromatin level, we next examined developmental changes in chromatin accessibility in mouse and NMR CMs. We generated ATAC-seq profiles from isolated CMs at P1 and adult stages for both species (Table 1). In mouse CMs, we identified 85,647 and 89,095 consensus accessible regions in P1 and adult stages, respectively (Fig. 11A). In contrast, NMR CMs exhibited 120,697 consensus peaks at P1 and 72,550 peaks in the adult stage (Fig. 11A). We then performed differential accessibility analysis to quantify chromatin remodeling during the P1-to-adult transition. Compared to P1, adult mouse CMs displayed 12,362 peaks with increased accessibility and 10,080 peaks with decreased accessibility (Fig. 11B and C). In contrast, adult NMR CMs showed 10,886 peaks with increased accessibility and only 3,698 peaks with decreased accessibility relative to P1 (Fig. 11B and C). Together, these results confirm that adult NMR hearts undergo a markedly attenuated closure of chromatin regions during the transition to adulthood, supporting the persistence of a neonatal regulatory landscape in adult NMR hearts.

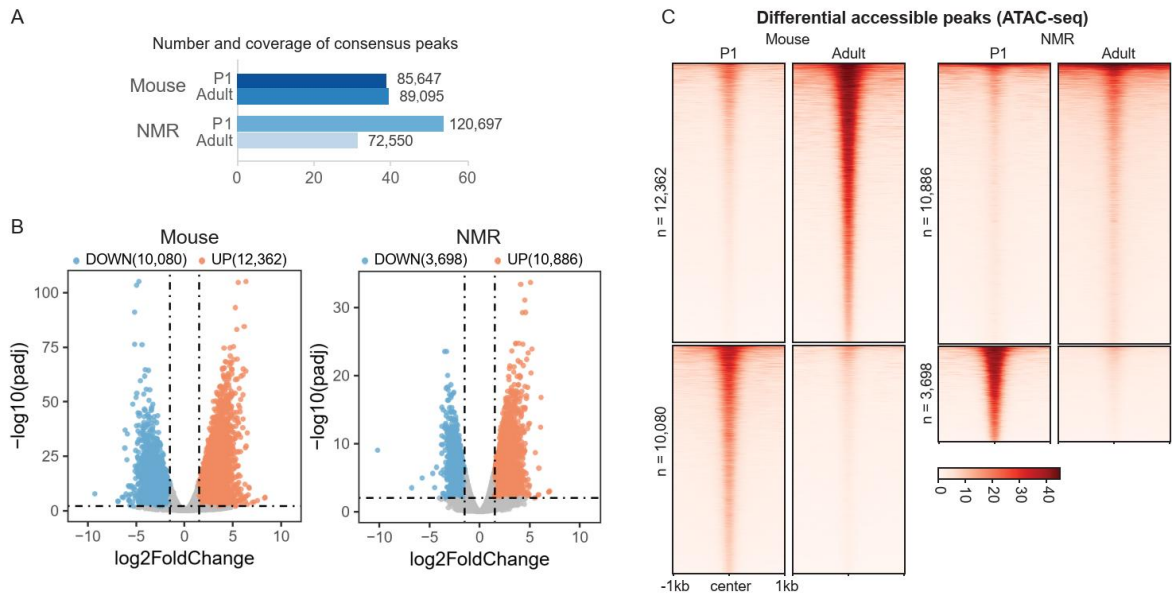


Fig. 11 Developmental dynamics of chromatin accessibility in mouse and naked mole-rat (*NMR*) cardiomyocytes.

(A) Numbers and coverage of consensus ATAC-seq peaks identified in cardiomyocytes (CMs) from mouse and NMR hearts at postnatal day 1 (P1) and adult stages. Bars indicate genomic coverage, with the x-axis shown in megabases (Mb). (B) Volcano plots showing differential chromatin accessibility between adult and P1 CMs in mouse (left) and NMR (right). Peaks with increased and decreased accessibility in adult relative to P1 are highlighted. (C) Heatmaps showing ATAC-seq signal intensity of differentially accessible peaks across P1 and adult CMs in mouse (left) and NMR (right), illustrating developmental changes in chromatin accessibility. Signals are scaled by FPKM.

4.1.2 Neonatal-like genes supporting regenerative potential in adult naked mole-rat hearts

To identify which genes and biological programs constitute the retained neonatal-like transcriptional state in adult NMR hearts, we intersected genes differentially expressed during mouse postnatal maturation with those differentially expressed between adult NMR and adult mouse hearts. This approach identified genes in adult NMR hearts that specifically retain neonatal-like expression patterns, rather than genes reflecting broader species-specific differences. Comparison of adult NMR and adult mouse hearts identified 1,626 upregulated and 1,702 downregulated differentially expressed genes (DEGs) in NMR hearts (Fig. 12A). To define genes contributing to the retained neonatal-like transcriptional state in adult NMR hearts, we focused on genes showing opposite developmental regulation in mouse heart maturation. Specifically, we defined upregulated neonatal-like genes as those elevated in adult NMR hearts relative to adult

mouse hearts but normally decreased during the mouse P1-to-adult transition. Conversely, downregulated neonatal-like genes were defined as genes that were reduced in adult NMR hearts relative to adult mouse hearts but were normally increased during mouse maturation. Using this approach, we identified 175 upregulated and 291 downregulated neonatal-like genes in adult NMR hearts (Fig. 12B; Table S1-2).

Gene Ontology (GO) analysis of upregulated neonatal-like genes revealed strong enrichment for cell cycle regulation processes, including cell division, DNA replication, chromosome segregation, and mitotic centrosome separation, indicating persistent expression of molecular programs typically associated with immature and proliferative CM states (Fig. 12C). In addition, pathways involved in DNA damage response, DNA repair, DNA recombination, and interstrand cross-link repair were significantly enriched, suggesting enhanced genome maintenance and stress-protective mechanisms in adult NMR hearts (Fig. 12C). In contrast, downregulated neonatal-like genes were primarily associated with metabolic processes, ion transport, and immune-related pathways, consistent with suppression of transcriptional programs linked to terminal CM maturation and adult metabolic specialization in NMR hearts (Fig. 12D).

Among the upregulated neonatal-like genes are *Cdc6*, *Cks2*, *Nek2/6*, and *Gpsm2*, which are involved in cell cycle progression and mitotic regulation. In addition, genes such as *Mcm3*, *ErbB2*, *Hmgb2*, and *Cks1b* have previously been implicated in promoting CM proliferation and maintaining an immature or plastic cellular state[54, 140–142]. However, despite retention of these neonatal-associated programs, our previous analyses detected no evidence of active CM proliferation in adult NMR hearts (Fig. 12E), suggesting that these genes do not drive overt cell-cycle progression in this context. Rather, their persistent expression may reflect maintenance of a partially immature or permissive molecular state associated with cellular plasticity, genome stability, and stress resilience.

Interestingly, we also identified reduced expression of *Ccng1* and *Ccnd1* in adult NMR hearts relative to adult mouse hearts. Both genes have previously been associated with CM multinucleation, in which increased expression promotes multinucleation and genetic inactivation reduces its extent[52, 53]. Consistent with this, our earlier analyses demonstrated that adult NMR hearts contain a higher proportion of mononucleated cardiomyocytes compared with mouse hearts (Fig. 7B). Together, these findings

suggest that adult NMR hearts retain selective neonatal-like transcriptional programs linked to developmental plasticity and incomplete terminal maturation, while remaining largely non-proliferative.

In addition, downregulated neonatal-like genes are associated with metabolic processes, particularly lipid-related processes, including triglyceride biosynthesis and utilization (*Dgat2*, *Gpam*, *Agpat2*, *Nr1h3* and *Lpl*) as well as lipid homeostasis (*Plin5*, *Fitm2* and *Nr1d2*). This confirmed our previous observations that lipid droplets were completely absent in adult NMR hearts[93]. Downregulated neonatal-like genes were also associated with fatty acid oxidation (FAO), including *Acsf6*, *Acox1*, *Ech1* and *Hadhb*). This consistent with our previous findings of reduced FAO activity in NMR hearts (Fig. 6). In contrast, glycogen-related genes were identified among the upregulated neonatal-like genes, including *Ppp1r3g*, *Stbd1*, and the liver isoform of glycogen phosphorylase, *Pygl* (Fig. 12F). This is in line with our previous results that NMR hearts exhibit a liver-like glycogen metabolism (Fig. 6). Notably, the muscle isoform of glycogen phosphorylase (*Pygm*) was also a downregulated neonatal-like gene (Fig. 12F). Taken together, these results indicate that neonatal-like genes retained in adult NMR hearts reflects a coordinated program integrating cell cycle regulation and metabolic reprogramming, which may contribute to maintaining a regeneration-permissive state.

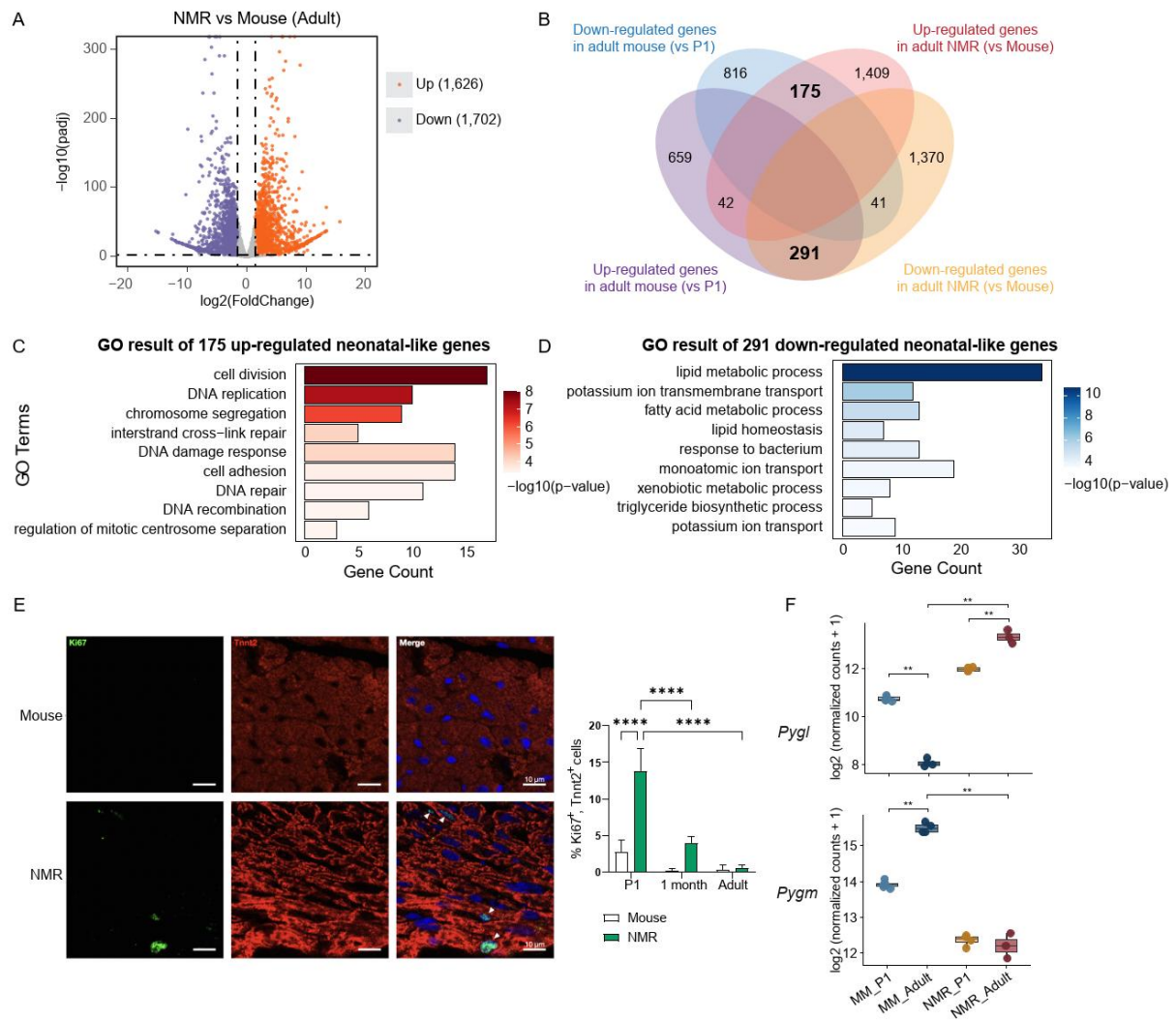


Fig. 12 Retention of a neonatal-like transcriptional profile in the adult naked mole-rat (NMR) heart.

(A) Volcano plot showing differentially expressed genes (DEGs) between adult NMR and mouse hearts. Upregulated and downregulated genes in NMR relative to mouse are highlighted. (B) Schematic illustrating the definition of neonatal-like genes. Neonatal-like genes were defined as those showing expression changes in adult NMR versus adult mouse hearts that were opposite to developmental changes from P1 to adult in the mouse. Overlapping of two DEG sets, 175 upregulated and 291 downregulated neonatal-like genes were identified. (C and D) Gene Ontology (GO) enrichment analysis of (C) upregulated and (D) downregulated neonatal-like genes. E, Representative immunostaining for Ki67 and Tnnt2 in mouse and NMR hearts at 1 month, along with quantification of Ki67⁺ and Tnnt2⁺ cardiomyocytes across different developmental stages, shown as bar plots. Ki67 marks proliferating cells; Tnnt2 labels cardiomyocytes. (F) Box plots showing normalized gene expression of glycogen phosphorylase genes Pygl and Pygm in adult and P1 mouse and NMR hearts. Each dot represents one biological replicate. ** indicates adjusted p-value < 0.01. Adjusted p-values (Benjamini-Hochberg correction) were calculated using DESeq2.

4.2 Regulatory and evolutionary mechanisms underlying adaptive cardiac phenotypes in naked mole-rats

4.2.1 Genome-wide annotation of cis-regulatory elements in adult hearts

To further investigate the transcriptional and epigenetic basis of the unique cardiac phenotype observed in adult NMRs compared with mice, we integrated multi-omics datasets from heart tissues of both species. To assess the underlying epigenetic landscape, we performed ChIP-seq for histone modifications in adult hearts: H3K4me3 (promoters), H3K4me1 (enhancers), H3K27ac (active signal), and H3K27me3 (repressed chromatin) (Table 2). These datasets enabled us to systematically characterize cis-regulatory elements and link epigenomic states to transcriptional outputs across species.

Table 2. Summary of ChIP-seq data from different histone marks in adult mouse and NMR hearts.

Histone marks	Tissue	Replicates(NMR/MM)
H3K4me1 (Enhancers)	Heart	3/3
H3K4me3 (Promoters)	Heart	3/3
H3K27ac (Active)	Heart	3/3
H3K27me3 (Repressed)	Heart	3/3

First, for the ChIP-seq data, we defined consensus peaks as those identified in at least two biological replicates and used them for all downstream analyses. In adult mouse hearts, we identified 55,841 active elements, 24,716 promoters, 42,164 enhancers, and 28,526 repressed regions. In adult NMR hearts, we detected 59,167 active elements, 26,239 promoters, 41,591 enhancers, and 40,395 repressed regions (Fig. 13A). Overall, the numbers of active regulatory elements, including promoters and enhancers, were comparable between the two species. However, NMR heart exhibited a substantially higher number of repressed elements, compared to the mouse heart. This observation is consistent with previous findings in fibroblasts, where NMRs were shown to have globally elevated levels of H3K27me3 relative to mice[143].

To assess the reliability of our NMRs datasets, we compared our consensus peaks with previously published datasets and found that approximately 85% of H3K27ac

elements, 98% of H3K4me3 elements, and 50% of H3K4me1 elements identified in previous studies were also detected in our analysis[144][50] (Fig. 13B). These results indicate high consistency for H3K27ac and H3K4me3 datasets and reveal additional regulatory elements not reported in previous studies (~35%), thereby expanding the regulatory landscape. Although only ~50% of the H3K4me1-marked regions overlapped with previous datasets, a substantial proportion of H3K4me1 peaks in both datasets overlapped with H3K27ac signals (74% in our data and 79% in public data; Fig. 13B and C). This overlap supports the overall reliability of our dataset and suggests that H3K27ac captures a comprehensive set of active regulatory elements, providing a solid basis for downstream analyses.

To define functional cis-regulatory elements, we classified H3K27ac peaks that overlapped with H3K4me3 as active promoters and those that overlapped with H3K4me1 as active enhancers. H3K27ac peaks that did not overlap with either H3K4me1 or H3K4me3 were categorized as putative enhancers in both mouse and NMR hearts. Based on this classification, we found that among the mouse H3K27ac consensus elements, 34.9% were active promoters, 25.5% were active enhancers, and 39.6% were putative enhancers (Fig. 13D, E). In NMR hearts, 33.4% were active promoters, 21.3% were active enhancers, and 45.3% were putative enhancers (Fig. 13D, E). The overall distribution of promoters and enhancers was comparable between these two species.

In this part, by integrating multiple histone modifications, we established a high-confidence map of functional cis-regulatory elements, providing a foundational framework for investigating transcriptional regulation and species-specific cardiac phenotypes.

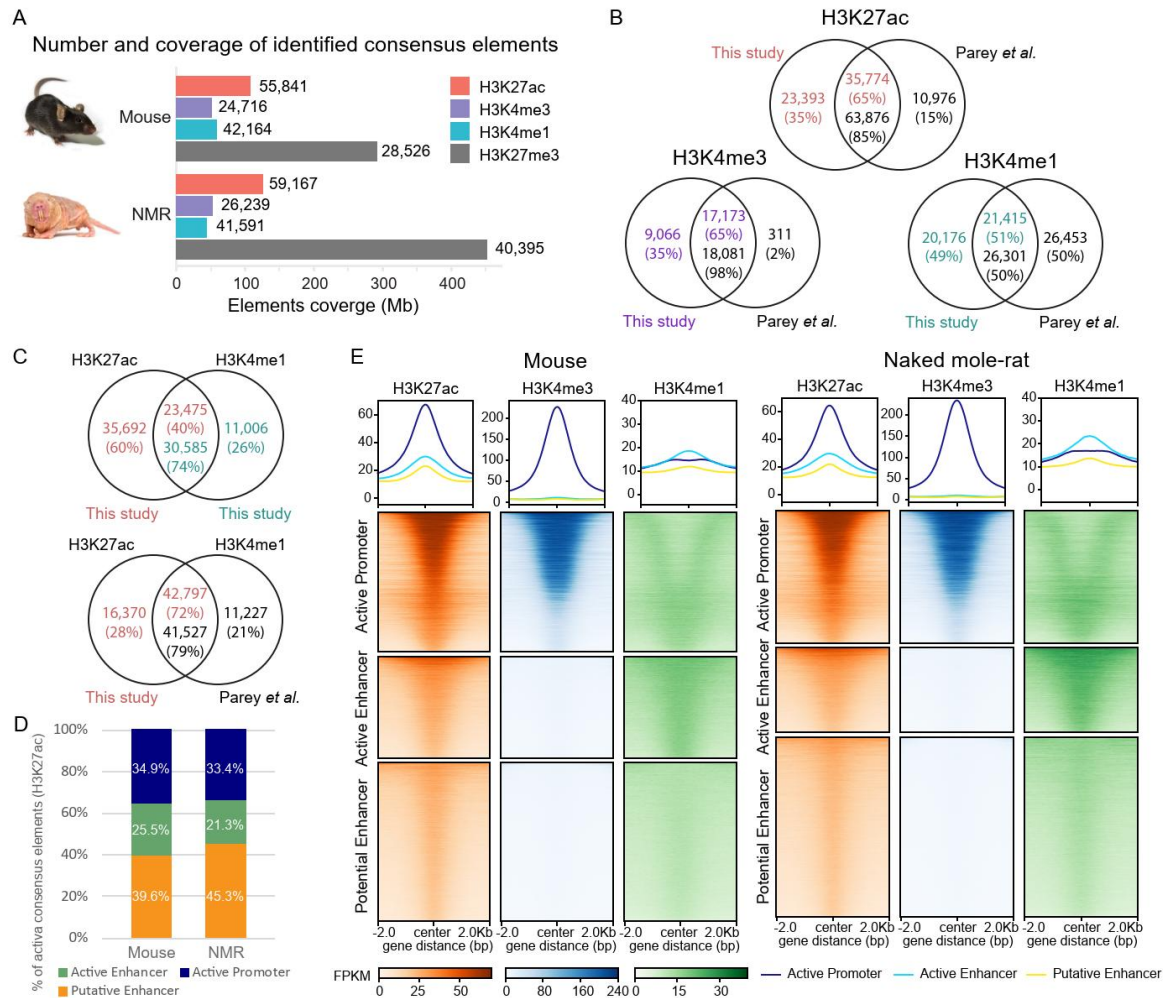


Fig. 13 Identification and classification of cis-regulatory elements in adult mouse and NMR hearts.

(A) Summary of coverage and number of ChIP-seq consensus peaks identified in mouse and NMR hearts for H3K27ac (active elements), H3K4me3 (promoters), H3K4me1 (enhancers), and H3K27me3 (repressed regions). Consensus peaks were defined as those identified in at least two biological replicates. (B) Venn diagram showing the overlap of H3K27ac, H3K4me3, and H3K4me1 peaks between this study and previously published datasets[50]. (C) Venn diagram showing the number and proportion of H3K4me1 peaks from our data and previous studies that overlapped with H3K27ac peaks. (D) Bar graph summarizing the proportions of H3K27ac-marked regions into active promoters (overlapping H3K4me3), active enhancers (overlapping H3K4me1), and putative enhancers (H3K27ac-only) in mouse and NMR hearts. (E) Heatmap showing normalized signal intensities (FPKM) of H3K27ac, H3K4me3, and H3K4me1 across the identified active promoters, active enhancers, and putative enhancers in both mouse and NMR hearts.

4.2.2 Identification of differentially regulated genes between naked mole-rat and mouse heart

To investigate how cis-regulatory elements contribute to interspecies differences in gene expression, we classified active regulatory peaks as orthologous or non-orthologous. Using the mouse genome as the reference, we performed pairwise genome alignments with the NMR genome. Active peaks that could be reciprocally aligned between the two genomes with a sequence identity greater than 30% were defined as orthologous peaks. In contrast, peaks that lacked such reciprocal alignment were categorized as non-orthologous, representing species-specific active regulatory regions. Based on this classification, we identified 52,857 orthologous peaks active in at least one species. We next classified the orthologous peaks into active promoters, active enhancers, and putative enhancers, using the same histone mark-based criteria described above. Notably, we found that the majority (~70%) of orthologous promoters were active in both mouse and NMR hearts. In contrast, most orthologous enhancers (~80%) were species-specific in their activity patterns (Fig. 14A). These results are consistent with recent datasets and indicate that promoters are highly conserved across species, whereas enhancers are mostly fast-evolving[144, 145]. Additionally, we detected 17,536 mouse-specific (non-orthologous) active peaks and 20,725 NMR-specific (non-orthologous) active peaks. To evaluate the evolutionary conservation of these regulatory elements, we assessed phastCons scores derived from 60-way vertebrate alignment. This analysis revealed that orthologous peaks exhibited significantly higher conservation levels compared to non-orthologous peaks, supporting the reliability of our classification strategy (Fig. 14B). Moreover, these results allowed us to identify both conserved and species-specific fast-evolving cis-regulatory elements, providing a foundation for exploring the regulatory basis of the unique cardiac phenotype in the NMR.

Next, focusing on the previously defined orthologous active regulatory elements, we performed differential analysis of H3K27ac signal between the two species. This analysis identified 12,029 elements with significantly higher H3K27ac signal in NMR (referred to as up-active peaks) and 11,912 peaks with lower H3K27ac signal in NMR compared with in mouse (down-active peaks) (Fig. 14C). These differential and species-specific peaks represent candidate cis-regulatory elements that may underlie transcriptional differences (Fig. 14C, D).

To further investigate how these regulatory elements influence gene expression, we assigned each differential or species-specific H3K27ac peak to its nearest gene and intersected these peak-associated genes with DEGs between adult NMR and mouse. We defined the resulting overlapping gene sets as Differentially Regulated Genes (DRGs). Specifically, up-DRGs were defined as up-DEGs located near either up-regulated H3K27ac peaks or NMR-specific peaks, while down-DRGs were down-DEGs located near down-regulated peaks or mouse-specific peaks (Fig. S1). Using this approach, we identified 1,052 up-DRGs and 1,080 down-DRGs, providing a set of high-confidence genes whose expression divergence is potentially driven by species-specific cis-regulatory activity (Fig. 14E, F).

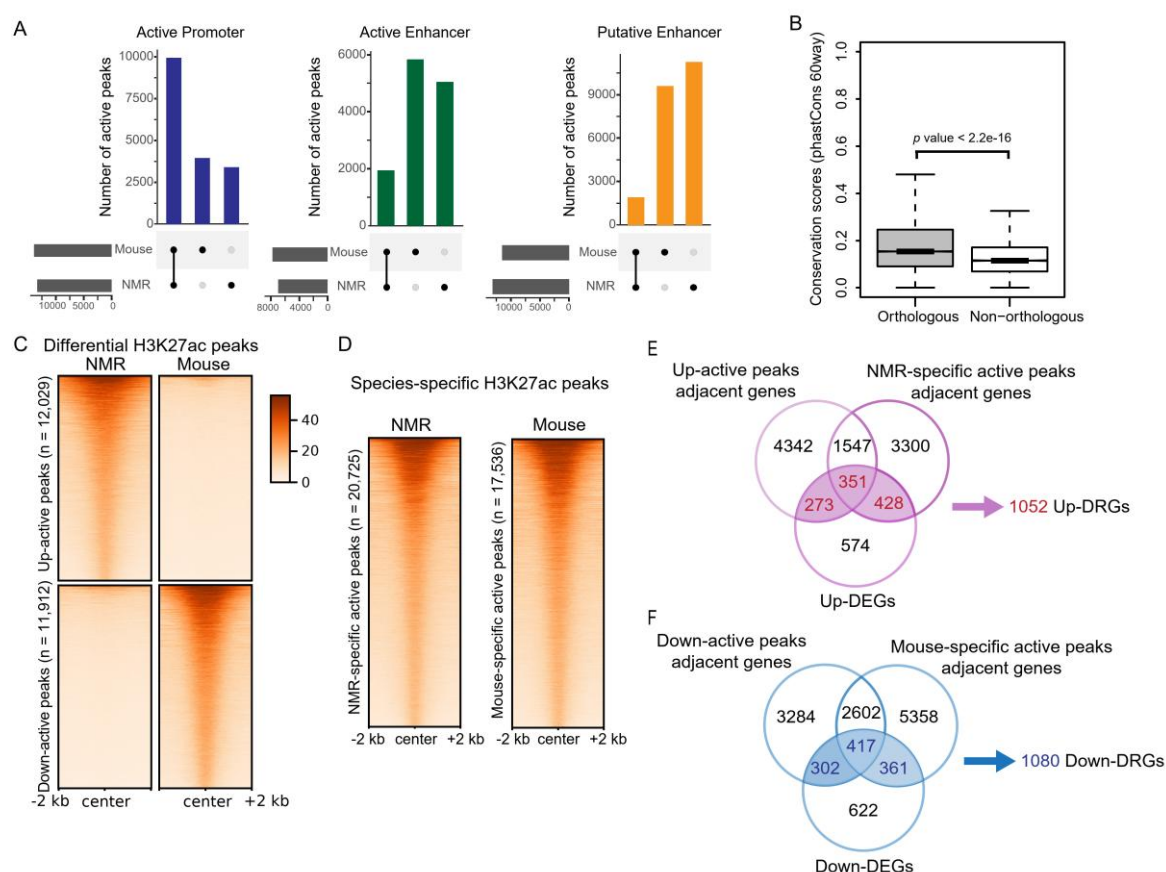


Fig. 14 Comparative analysis of cis-regulatory activity and gene expression between adult mouse and NMR hearts.

(A) Activity of orthologous promoters and enhancers across species. Upset plots show the number of orthologous active promoters (blue), active enhancers (green), and putative enhancers (orange) across two species or species-specific. (B) Conservation analysis of orthologous and non-orthologous H3K27ac peaks using 60-way phastCons scores. Orthologous peaks show significantly higher sequence conservation than non-orthologous peaks (Wilcoxon test). (C) Heatmap showing differential H3K27ac signal at orthologous regulatory elements between NMR and mouse. (D) Heatmap showing

H3K27ac signal of species-specific H3K27ac peaks identified in NMR and mouse hearts. (E and F) Venn diagrams showing overlap between DEGs and genes associated with differential or species-specific H3K27ac peaks for defining up- (E) and down-regulated (F) DRGs.

4.2.3 Cytoskeletal remodeling in adult NMR heart

To gain insights into the biological functions associated with DRGs, we performed GO enrichment analyses separately for up- and down-DRGs. The top 15 enriched biological processes revealed that up-DRGs were significantly associated with cytoskeletal organization and metabolism (Fig. 15A). Specifically, enriched terms included actin filament organization, protein polymerization, and negative regulation of cytoskeleton organization, indicating potential roles in cellular structural remodeling. Several terms related to organelle organization and cell morphogenesis were also enriched, suggesting involvement in reshaping CM structure (Fig. 15A). Moreover, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis identified significant enrichment in the cytoskeleton in muscle cells pathway (Fig. 15A). Surprisingly, genes associated with 3,393 NMR fast-evolving elements among 100-way conserved mammalian non-exonic elements were also enriched in pathways linked to cytoskeletal organization and adherens junction (Table 3), further supporting a role for regulatory rewiring in structural remodeling of the NMR heart.

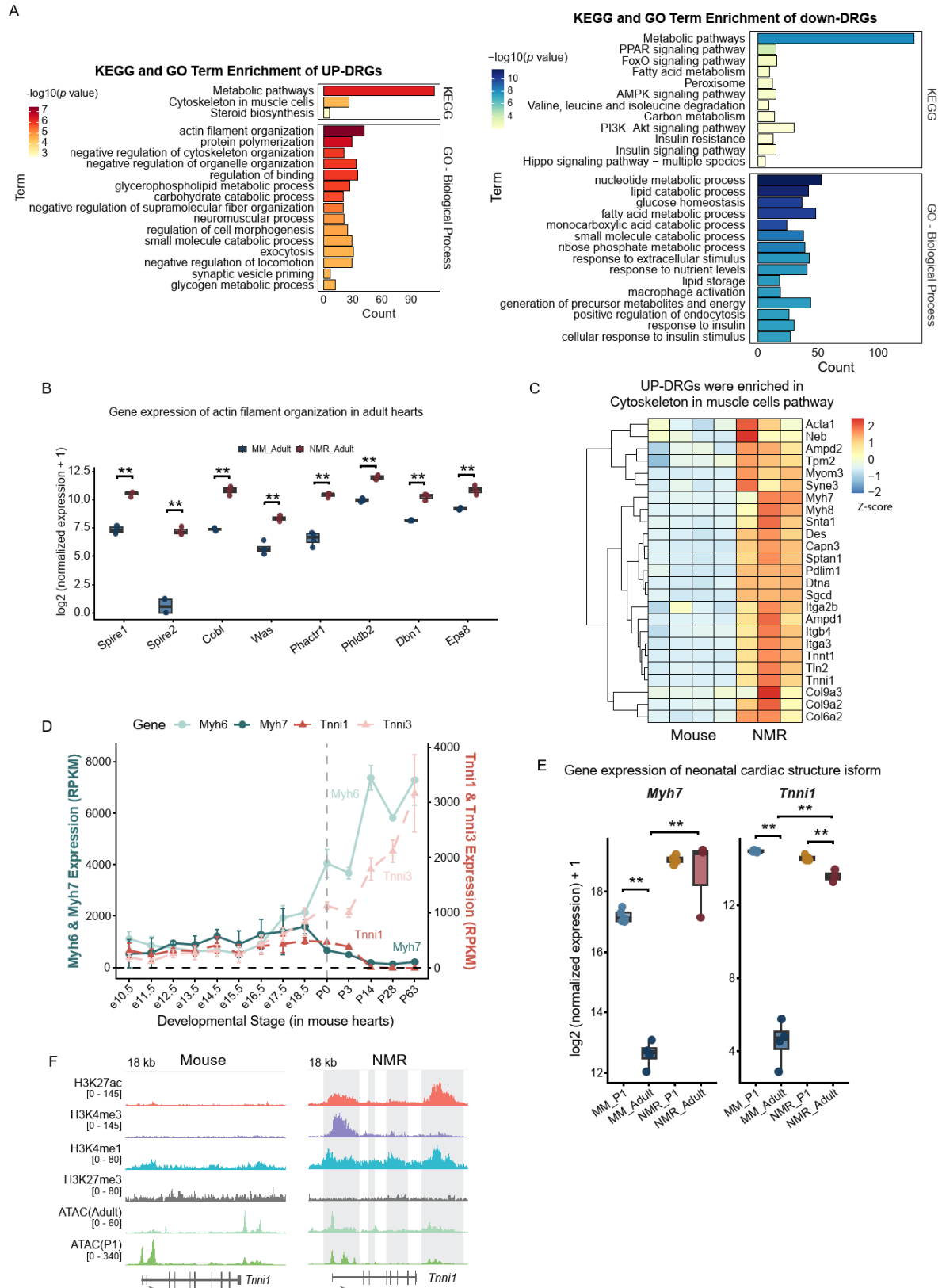


Fig 15. Expression and active regulatory landscape of slow skeletal and neonatal sarcomere genes in adult naked mole-rat hearts.

(A) Gene Ontology and KEGG pathway enrichment analysis of up- (left panel) and down- (right panel) DRGs in the adult NMR compared to adult mouse heart. Pathways with p -value < 0.01 and the top 15 enriched biological process terms are shown. (B) Box plots showing normalized gene expression of actin filament organization genes in adult mouse and NMR hearts. Each dot represents one biological replicate. ** indicates adjusted p -value < 0.01 . Adjusted p -values (Benjamini-Hochberg correction) were calculated using DESeq2. (C) Heatmap showing the relative expression in mouse and NMR heart of genes which significantly enriched in Cytoskeleton in muscle cell pathway. (D) Heart developmental gene expression trajectories of neonatal (*Myh7* and *Tnni1*) and adult (*Myh6* and *Tnni3*) cardiac structural isoforms in mouse hearts (data from Cardoso-Moreira et al.)[146]. (E) Box plots showing normalized gene expression of two neonatal structural genes (*Tnni1* and *Myh7*) in P1 and adult mouse and NMR hearts. Each dot represents one biological replicate. ** indicates adjusted p -value < 0.01 . Adjusted p -values (Benjamini-Hochberg correction) were calculated using DESeq2. (F) Epigenetic profiles of *Tnni1* loci in mouse and NMR hearts, including ChIP-seq signals for H3K27ac, H3K4me3, H3K4me1, and H3K27me3, as well as ATAC-seq signals in adult mouse and NMR hearts. ATAC-seq signals from P1 hearts in both species are also shown. H3K27ac-positive regions are indicated by grey rectangles. Arrows indicate transcriptional direction.

Table 3. KEGG pathway result of genes associated with 3,393 NMR fast-evolving highly conserved elements. (FDR < 0.05)

Term	Gene Count	p Value	FDR
Rap1 signaling pathway	28	1.99E-05	0.006018
Arrhythmogenic right ventricular cardiomyopathy	15	1.42E-04	0.021429
Adherens junction	15	2.95E-04	0.029687
Cytoskeleton in muscle cells	26	4.97E-04	0.031706
Axon guidance	22	5.25E-04	0.031706
PI3K-Akt signaling pathway	35	8.87E-04	0.044648

Among these cytoskeleton-related Up-DRGs, many are primarily involved in actin filament organization and sarcomeric structures (Fig. 15A). Within the actin-related genes, *Spire1/2*, *Cobl* and *Was* are mainly associated with actin nucleation[147–149], whereas other genes, including *Phactr1*, *Phldb2*, *Dbn1* and *Eps8*, are involved in the dynamic regulation of actin organization (Fig. 15B). Notably, *Spire1*, *Phactr1* and *Phldb2* are also identified as genes with NMR fast-evolving elements. Their enrichment suggests that adult NMR hearts retain a cytoskeletal remodeling program that may support CM structural plasticity. Notably, this pattern is consistent with previous studies showing that *ErbB2*-YAP activation promotes EMT-like CM remodeling, including

cytoskeletal reorganization, junction remodeling, and migration during cardiac regeneration[150].

In addition, *Tnni1*, *Myh7*, *Tnnt1* and *Tpm2* are associated with sarcomere architecture, playing critical roles in maintaining sarcomere integrity and regulating muscle contraction (Fig. 15C). Notably, during mouse heart development, CMs undergo an isoform switch in contractile proteins, transitioning from neonatal isoforms such as *Tnni1* and *Myh7* to adult isoforms *Tnni3* and *Myh6* (Fig. 15D). In contrast, adult NMR hearts express both adult isoforms and retain neonatal isoforms, as both fetal isoform genes were identified as NMR neonatal-like genes (Fig. 15E; Table S1), indicating a partial preservation of immature sarcomeric features. This is consistent with our previous observation that adult NMR CMs resemble the morphological features of neonatal mouse CMs (Fig. 7A). Consistently, analysis of chromatin accessibility revealed an active promoter and three enhancers at the *Tnni1* locus in the adult NMR heart, whereas these regulatory elements were not detected in the adult mouse heart. Notably, open chromatin peaks in the promoter region were observed in both NMR and P1 mouse hearts (Fig. 15F), consistent with previous reports showing that H3K27ac signals at the *Tnni1* locus are lost during mouse heart maturation[151]. Together, these findings suggest that neonatal sarcomeric regulatory programs remain epigenetically active in the adult NMR heart. In addition, *Tnni1* and *Tnnt1* encode slow skeletal troponin isoforms[152], *Myh7* encodes the slow myosin heavy chain isoform[153], and *Tpm2* encodes β -tropomyosin, a skeletal muscle tropomyosin isoform enriched in slow muscle fibers[154]. Together, the sustained expression of these genes suggests the retention of a slow-type contractile program, which may be consistent with the relatively low basal cardiac function observed in NMR hearts.

Taken together, these results reveal that cytoskeletal remodeling in the adult NMR heart is driven by changes in cis-regulatory element activity, suggesting regulatory rewiring that underlies the coordinated activation of actin- and sarcomere-associated gene programs and is associated with neonatal-like cardiac structural features and regenerative capacity.

4.2.4 Cis-regulatory activity causing shifts in metabolic-related gene expression in NMRs

Metabolic remodeling from glycolysis to FAO is a hallmark of cardiac maturation during postnatal development and is accompanied by the loss of regenerative capacity. Conversely, reversal of this metabolic transition has been shown to promote CM proliferation and cardiac regeneration[68, 74]. Consistent with this, KEGG results of DRGs reveal that both Up- and Down-DRGs are significantly enriched in metabolic pathways (Fig. 15A), suggesting substantial differences in metabolic processes in the hearts of these two species. Up-DRGs were significantly enriched in pathways related to carbohydrate and glycogen metabolism, consistent with the glycogen accumulation phenotype reported in our previous study[93], while Down-DRGs were mainly enriched in lipid and fatty acid metabolic processes (Fig. 15A). These findings suggest that the metabolic program of adult NMR heart is remodeled compared to that of mouse, relying more on glycolysis rather than FAO for energy production, which may contribute to the maintenance of regenerative potential.

Among Up-DRGs, glycogen-related genes such as *Pygl*, *Ppp1r3b*, *Ppp1r3c*, *Ppp1r3g* and *Stbd1* were identified (Fig. 16A). In our previous study, we showed that NMR hearts exhibit a liver-like glycogen metabolism. Unlike adult mouse hearts, which primarily rely on the muscle-type glycogen phosphorylase (*Pygm*), NMR hearts predominantly express the liver-type isoform (*Pygl*)[93]. Consistent with this, in this study, *Pygl* and *Pygm* were identified as upregulated and downregulated neonatal-like genes in NMR, respectively (Fig. 12F), and were also classified as Up-DRGs and Down-DRGs, respectively (Fig. 16A). Consistent with these observations, epigenomic data revealed that *Pygl* promoter is markedly more active in NMR than in mice, accompanied by the presence of three NMR-specific enhancers across the gene body, which are not detected even in neonatal mouse hearts (Fig. 16B). Additionally, *Stbd1*, a key player in glycogen transport to lysosomes, is also an up-regulated neonatal-like gene (Table S1). Although an accessible promoter region was observed across both developmental stages and species, adult mouse hearts exhibited a bivalent promoter state, whereas increased promoter activity together with enhanced distal regulatory activity was observed in adult NMR hearts (Fig. 16 A and C). Interestingly, our previous results showed that disruption of glycogen metabolism using α -glucosidase inhibitor acarbose resulted in a larger injury after ischemia reperfusion[93], further supporting the notion that glycogen metabolic reprogramming observed in NMR hearts may serve

as an adaptive cardioprotective mechanism. In addition, *Pfkfb* and *Pfkfb2*, key regulators of glycolytic flux, were both identified as Up-DRGs (Fig. 16A). Notably, deficiency of *Pfkfb* has been reported to impair glycolytic activity and reduce CMs proliferation in mouse embryonic CMs and immature human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs)[155]. Together, these findings suggest that NMR-specific cis-regulatory elements promote the expression of glycogen-related genes, thereby enabling NMR hearts to rely on a glycogen-dependent glycolytic metabolism, which may be associated with regenerative potential.

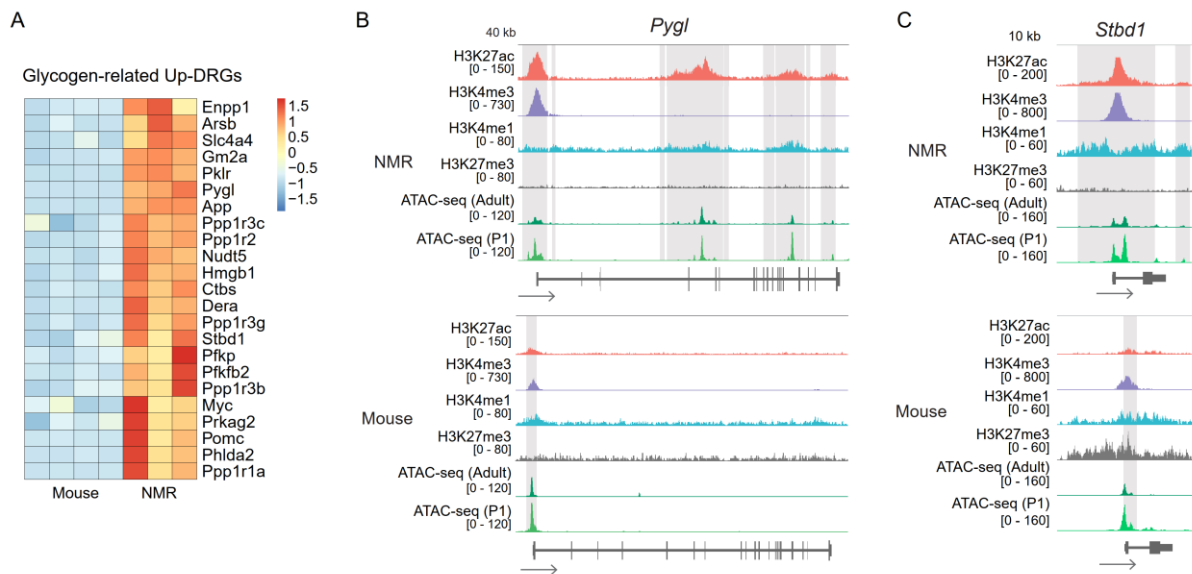


Fig 16. Epigenomic and transcriptional activation of glycogen metabolism-related genes in NMR hearts.

(A) Heatmap showing the relative expression of Up-DRGs involved in glycogen-related processes in adult mouse and NMR hearts. B-C, Epigenomic landscapes at the *Pygl* (B) and *Stbd1* (C) loci, including ChIP-seq signals for H3K27ac, H3K4me3, H3K4me1, and H3K27me3, as well as ATAC-seq signals in adult mouse and NMR hearts. ATAC-seq signals from P1 hearts in both species are also shown. H3K27ac-positive regions are indicated by grey rectangles. Arrows indicate transcriptional direction.

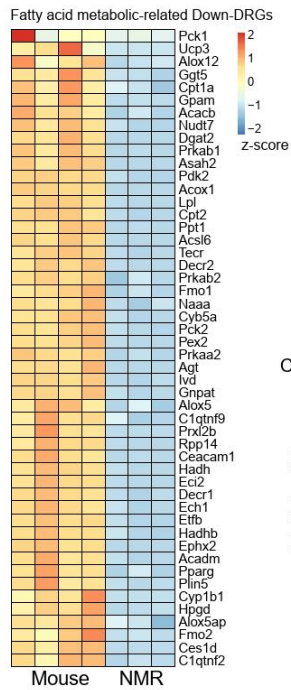
In contrast, Down-DRGs were enriched in pathways associated with lipid metabolism and homeostasis, including lipid storage and fatty acid metabolic processes (Fig. 15A). Consistent with this, genes involved in triglyceride biosynthesis, utilization, and lipid homeostasis, such as *Dgat2*, *Gpm*, *Agpat2*, *Nr1h3*, *Lpl*, *Plin5* and *Fitm2*, were identified as downregulated neonatal-like genes in NMR and were also classified as Down-DRGs (Fig. 17A and B; Table S2). Notably, *Dgat2*, *Gpm*, and *Agpat2* encode

key enzymes involved in triglyceride synthesis. In addition, *Plin3*, a lipid droplet-associated protein, was also identified as a Down-DRG, and its knockdown has been reported to reduce lipid accumulation[156]. Furthermore, *Pparg*, a key regulator of fatty acid uptake, triglyceride synthesis, and lipid droplet formation[157], was identified as a Down-DRG. These findings further support our observation that lipid droplets are absent in adult NMR hearts, while lipid droplets are significantly reduced in neonatal mouse hearts compared to adult mouse hearts (Fig. 17C). This pattern is consistent with the gene expression and epigenetic changes observed in our study (Fig. 17 B and D). Moreover, other Peroxisome Proliferator-Activated Receptors (PPARs) and their coactivators, *Ppargc1a* and *Ppargc1b*, are very important regulators of mitochondrial oxidative metabolism. Consistent with this, we found that, in addition to *Pparg*, *Ppard* and *Ppargc1a* were also downregulated to varying degrees in NMR hearts (Fig. 17E). In line with this, many Down-DRGs were associated with mitochondrial and peroxisomal FAO, including key enzymes such as *Cpt1a*, *Cpt2*, *Acadm*, *Decr1/2*, *Ech1*, *Eci2*, *Hadh*, *Acox1* and *Hadhb* (Fig. 17A and F). Consistent with these observations, previous studies have shown that *ppargc1a* mutant hearts exhibit reduced mitochondrial oxidative metabolism accompanied by increased glycolytic activity and enhanced CM proliferation[69]. Similarly, cardiac-specific knockout of *Cpt1* inhibits FAO, thereby promoting CM proliferation and improving cardiac functional recovery following injury[68, 158].

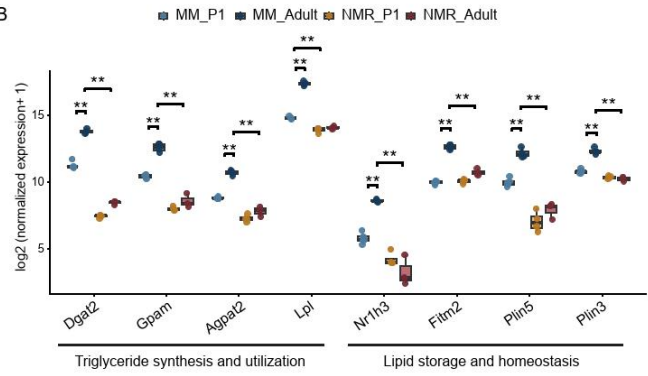
Downregulation of FAO is typically expected to reduce fatty acid utilization, potentially leading to fatty acid accumulation and lipotoxicity. However, our previous findings showed that fatty acid levels in NMR hearts are comparable to those in mouse hearts, indicating that fatty acids do not accumulate despite reduced FAO[93]. In addition, reduced triglyceride biosynthesis and the absence of lipid droplet accumulation in NMR hearts further suggest that fatty acids are not stored as neutral lipids. Notably, several Up-DRGs were directly involved in glycerophospholipid metabolism, including genes associated with phospholipid synthesis (*Acs13*, *Gpat3*, *Agpat5* and *Lpin2*), remodeling (*Lpcat4* and *Pla2g4b*) and phosphoinositide signaling (*Pi4k2b*, *Pip5k1b*, *Inpp5f*, *Pik3cb*, *Pik3c2a*) (Fig. 15A and Fig 17G). Together, these findings suggest that fatty acids are redirected toward phospholipid anabolism in NMR hearts, potentially supporting membrane remodeling and cellular adaptation.

Collectively, these findings indicate that metabolic reprogramming occurs from FAO to a more neonatal, glycogen-dependent glycolytic process in adult NMR hearts. Furthermore, fatty acid utilization is redirected from lipid storage, triglyceride biosynthesis, and FAO toward glycerophospholipid metabolism. Together, these changes reflect adaptive cis-regulatory remodeling of energy metabolism and may be associated with the regenerative potential of NMR hearts.

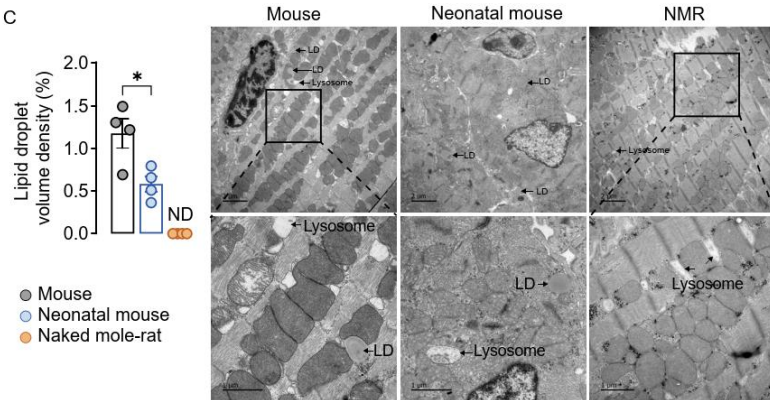
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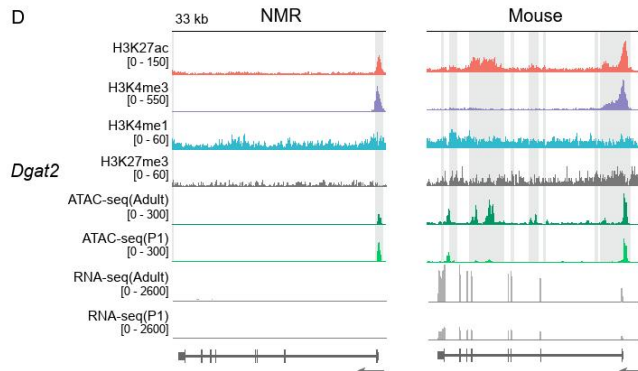
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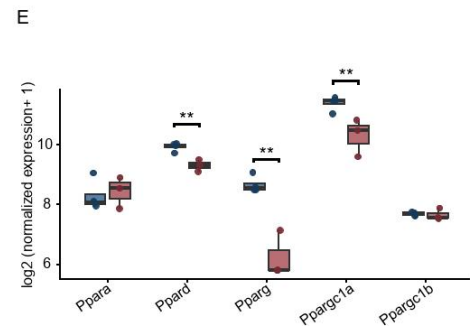
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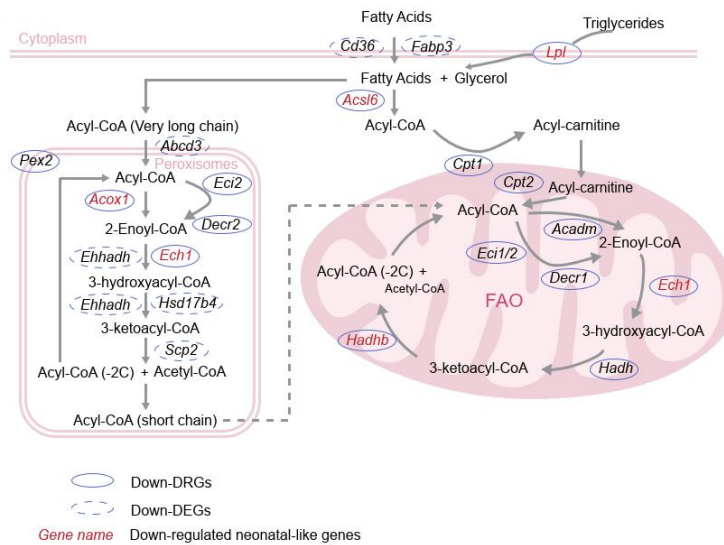
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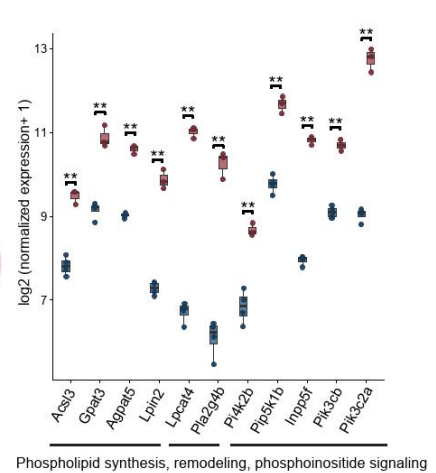


Fig 17. Downregulation of fatty acid oxidation and fatty acid metabolic remodeling in NMR hearts.

(A) A heatmap showing the relative expression of fatty acid metabolism-related Down-DRGs in adult mouse and NMR hearts. (B) Box plots showing normalized gene expression of Up-DRGs (most of which are also up-regulated neonatal-like genes) related to triglyceride biosynthesis, utilization, and lipid homeostasis in P1 and adult mouse and NMR hearts. Each dot represents one biological replicate. (C) Quantification of % lipid droplet volume density in naked adult and neonatal (P1) mouse and adult NMR hearts and representative transmission electron microscopy images, scale bar = 2 μ M top panel, 1 μ M bottom panel. Arrows point to representative lipid droplets (LD) and lysosomes ($n = 52$ -62 individual images for $n = 4$ biological replicates). (D) Epigenomic landscapes at *Dgat2* loci, including ChIP-seq signals for H3K27ac, H3K4me3, H3K4me1, and H3K27me3, as well as ATAC-seq signals in adult mouse and NMR hearts. ATAC-seq signals from P1 hearts in both species are also shown. H3K27ac-positive regions are indicated by grey rectangles. Arrows indicate transcriptional direction. (E) Bar plots showing gene expression (TPM) of PPARs (*Ppara*, *Ppard* and *Pparg*) and their coactivators (*Ppargc1a* and *Ppargc1b*) in adult mouse and NMR hearts. (F) Diagram of the fatty acid β -oxidation pathway, highlighting genes that are downregulated in NMR hearts. Genes enclosed by solid blue ellipses represent Down-DRGs, whereas those indicated by dashed ellipses correspond to genes with adjusted p -value < 0.01 and either $-1.5 < \log_2\text{FoldChange} < -1$ or $\log_2\text{FoldChange} < -1.5$ but not annotated as the nearest genes to down-active peaks. Gene names highlighted in the diagram denote genes that are also identified as downregulated neonatal-like genes. (G) Barplots showing gene expression (TPM) of Up-DRGs related to glycerophospholipid metabolism in adult mouse and NMR hearts. Each dot represents one biological replicate. ** indicates adjusted p -value < 0.01 . Adjusted p -values (Benjamini-Hochberg correction) were calculated using DESeq2.

4.2.5 Cis-regulatory elements recruit differential TFs driving differential functional output

To investigate whether differential activity of regulatory elements between NMR and mouse hearts is driven by transcription factor (TF) binding, we performed motif enrichment analysis on differentially active peaks. To ensure that the signal originated from CMs, differential H3K27ac peaks were intersected with CM ATAC-seq peaks (Fig. 18A). We then classified the differential peaks into four categories (2, 582 NMR more-active accessible promoters, 4,728 NMR more-active accessible enhancers, 2,432 mouse more-active accessible promoters, and 4,061 mouse more-active accessible enhancers) and conducted motif enrichment separately for each set.

Across all four peak sets, motifs of the myocyte enhancer factor-2 (MEF2) family, including Mef2a, Mef2b, Mef2c, and Mef2d, were significantly enriched (Table S1).

MEF2, a core cardiac transcription factor family, are well-known regulators of cytoskeletal organization and energy metabolism in heart development and disease[79]. For the NMR more-active accessible promoters, BHLHE40 (Basic Helix-Loop-Helix Family Member E40) and BHLHE41 binding motifs were enriched (Fig. 18B; Table S1). BHLHE40/41 are key regulators of the hypoxia response pathway and are known to be upregulated and strongly selected under hypoxic conditions, which may contribute to the adaptation of NMRs to their hypoxic subterranean environment[159, 160]. Consistently, Bhlhe41 showed upregulated expression in NMR hearts. For the NMR more-active accessible enhancers, AP-1 (Activator Protein-1) was enriched (Fig. 18C; Table S3). AP-1 has been shown to promote sarcomere disassembly, CM proliferation, and dedifferentiation during injury response by regulating target gene expression[60, 161]. The AP-1 target gene *fbx/22* is also an UP-DRG in NMR hearts. Its overexpression can also promote sarcomere disassembly[60]. For the mouse more-active accessible enhancers, GRE (Glucocorticoid Response Element), ESRRG (Estrogen-Related Receptor Gamma), and ESRRB (Estrogen-Related Receptor Beta) binding motifs were enriched (Fig. 18D; Table S1). Notably, GRE is recognized and bound by glucocorticoid receptor (GR), GR and ERRs both contribute to the metabolic transition from carbohydrate to FAO, and their inhibition or knockdown facilitates cardiac regenerative capacity[86, 162, 163]. These motif enrichment patterns suggest that distinct protein complexes may be recruited to cis-regulatory elements in NMR and mouse hearts, thereby dictating divergent downstream gene expression programs that underlie species-specific differences in metabolic remodeling and cardiac adaptation.

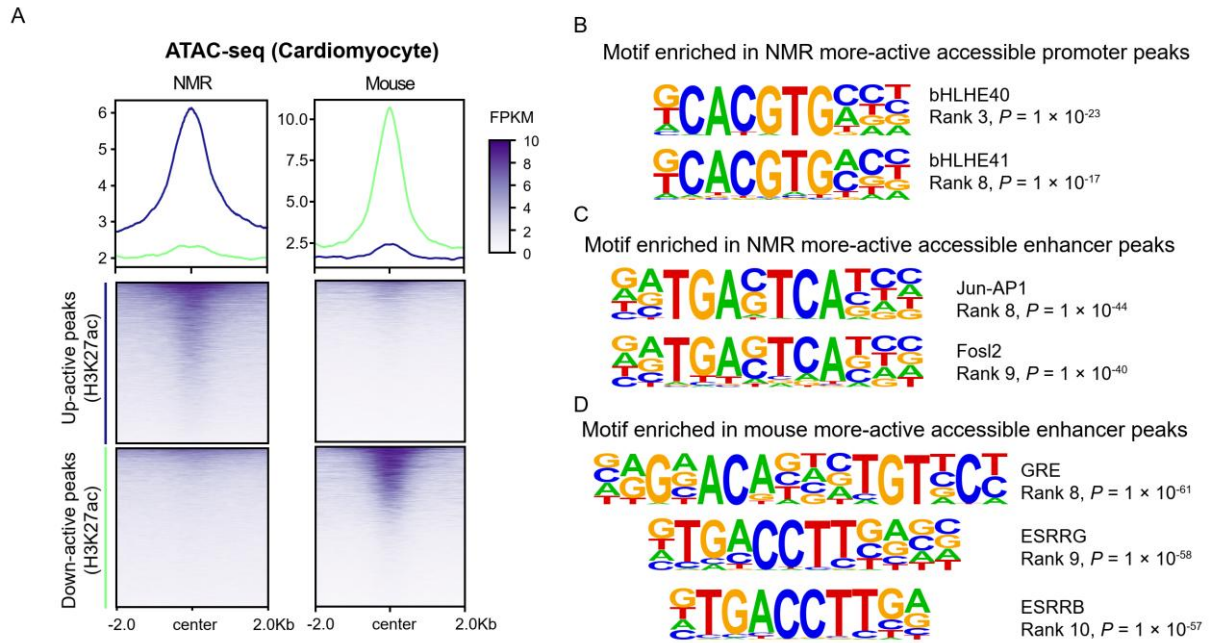


Fig 18. Motif enrichment analysis of differential cis-regulatory elements between NMR and mouse hearts.

(A) Heatmaps depicting FPKM of the ATAC-seq signal at the Up-active peaks (blue) and at the Down-active peaks (green). (B) Significantly enriched binding motifs of 2,582 NMR more-active accessible promoters. (C and D) Significantly enriched binding motifs of 2,432 mouse more-active accessible promoters (C) and 4,061 mouse more-active accessible enhancers (D).

4.2.6 ERRs directly drive metabolic and contractile changes in heart

The estrogen-related receptor (ERR) family, which includes $ERR\alpha$ (*Esrra*), $ERR\beta$ (*Esrrb*), and $ERR\gamma$ (*Esrrg*), has been implicated in previous studies as a key regulator of cardiac maturation. In particular, $ERR\alpha$ and $ERR\gamma$ play crucial roles in orchestrating energy metabolism and the contractile machinery during heart development[83, 85, 86]. Given the significant motif enrichment of ERR family in mouse more-active accessible enhancers, along with downregulation of their gene expression in the NMR hearts (Fig. 18E and 19A), we further explored the potential role of ERRs in driving interspecies differences in cardiac function. Notably, ERR expression progressively increases during postnatal cardiac maturation in mice, reflecting the metabolic reprogramming associated with the transition from fetal to adult cardiac function (Fig. 19B). In addition, heat maps showing H3K27ac signals at ERR motif-containing down-regulated peaks in mouse hearts and the corresponding orthologous regions in NMR hearts revealed substantially higher H3K27ac enrichment in mouse than in NMR (Fig.

19C). To further examine chromatin accessibility at these ERR motif sites, we applied the NucleoATAC algorithm to infer nucleosome occupancy. This analysis showed that ERR motif-containing regions within the down-regulated peaks were globally depleted of nucleosomes in mouse hearts, consistent with a more accessible chromatin state (Fig. 19D). To further investigate potential evolutionary divergence, we aligned ERRs protein sequences across 18 mammalian species and identified NMR-specific amino acid substitutions in all three ERR family members, including three substitutions in ESRRA and two substitutions each in ESRRB and ESRRG (Fig. 19E). In addition, we identified 29 NMR fast-evolving elements within the *Esrrg* gene body (Fig. 19F; Table S4). In parallel, we performed dN/dS analysis in ESRRG across 15 mammalian species and found that, although all branches are under strong purifying selection, the NMR lineage shows an increased accumulation of nonsynonymous substitutions (Fig. 19G). Together, these NMR-specific sequence variations and evolutionary features indicate potential functional divergence of ERRs in NMR hearts, in association with their distinct metabolic and contractile features.

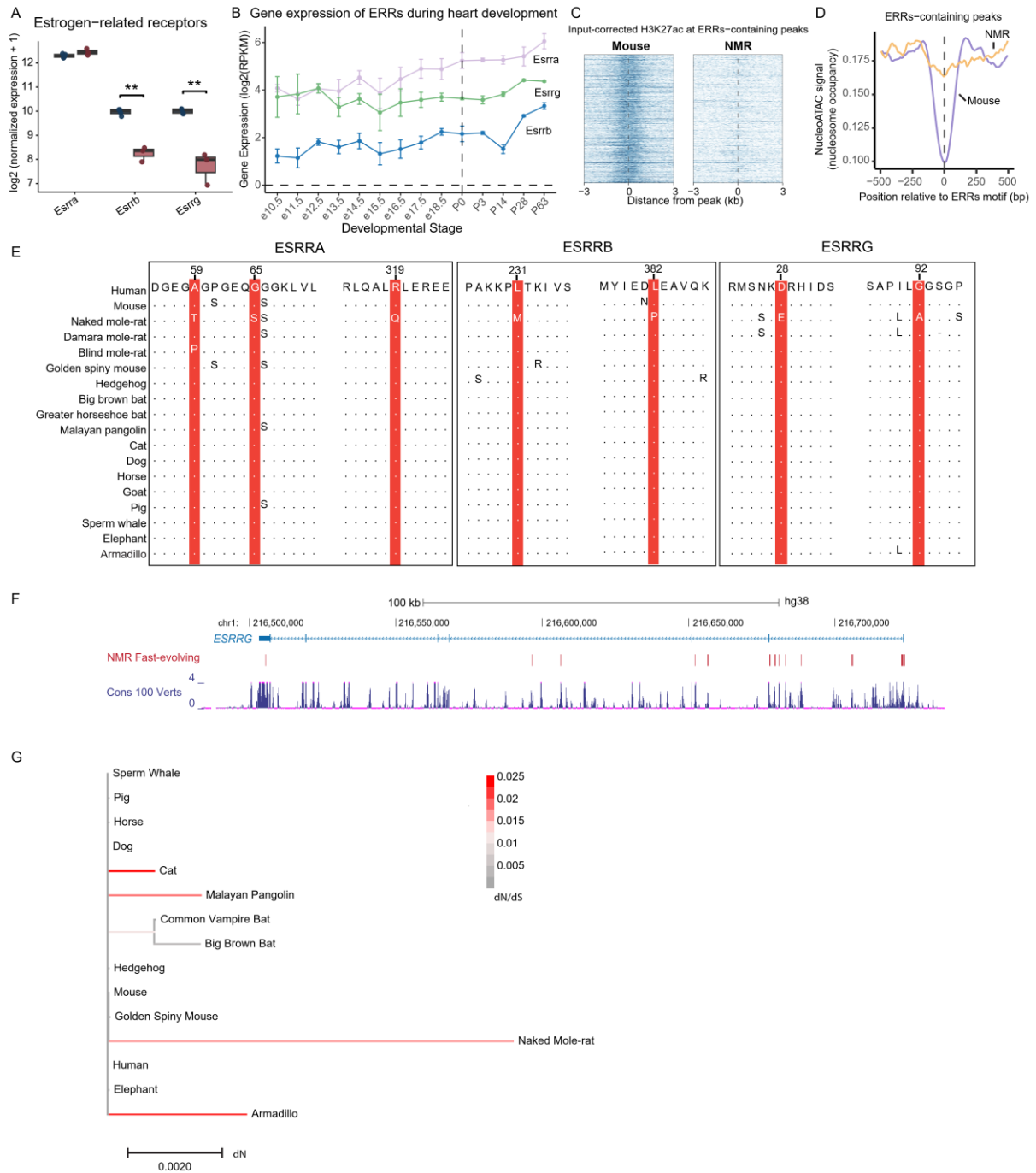


Fig 19. Regulatory, expression, and evolutionary features of ERR family genes in mouse and naked mole-rat (NMR) hearts.

(A) Expression levels of ERRs (*Esrra*, *Esrrb* and *Esrrg*) in NMR and mouse hearts. Each dot represents one biological replicate. ** indicates adjusted p -value < 0.01 . Adjusted p -values (Benjamini-Hochberg correction) were calculated using DESeq2. (B) Developmental expression dynamics of ERRs during heart development in mice. (C) H3K27ac signal for ERR motif peaks in down-regulated peaks. (D) Nucleosome signal for all ERR motif peaks in down-regulated peaks. (E) ERR protein sequence alignment across 18 mammalian species with NMR-specific amino acid substitutions highlighted by red rectangles. (F) 29 NMR fast-evolving elements in the region of *ESRRG* gene body. The elements in red

indicate fast-evolving elements. Blue bars indicate conserved regions in 100 vertebrates. (G) Non-synonymous substitution rate (dN) of ESRRG in 15 mammalian species. The dN shows that the gene in NMR has accumulated many mutations. The branch lengths represent dN values, while the different dN/dS ratios are shown in different colors.

To further explore species-specific differences in ERR target regulation, we leveraged transcription factor footprinting analysis. By intersecting ATAC-seq peaks harboring an ERRs motif with Up- and Down-DRGs, we identified 171 ERR target genes (genes with ERRs footprints) overlapped with Up-DRGs (Fig. 20A). In contrast, 197 ERR target genes overlapped with Down-DRGs (Fig. 20A). Given that ERRs are key transcriptional activator of mitochondrial and fatty acid oxidation programs, the enrichment of ERRs-containing regions among Down-DRGs is consistent with ERR driving the postnatal metabolic shift toward oxidative, fatty acid-based metabolism during cardiac maturation[83, 86]. Conversely, the relative absence of ERR engagement in NMR suggests that reduced ERR activity contributes to the attenuation of cardiac maturation programs in this species. Consistent with this, GO analysis of ERR-associated Down-DRGs showed enrichment for FAO and lipid storage and metabolism (Fig. 20B). In contrast, ERR-associated Up-DRGs were enriched for cytoskeletal organization and glycolytic metabolic processes (Fig. 20C), suggesting that ERR-associated regulatory elements are linked to both metabolic and structural gene programs.

To assess whether these DRGs represent bona fide ERR targets, we leveraged a curated set of direct ERR-activated and -suppressed genes derived from the intersection of postnatal CM-specific ERR α / γ knockdown RNA-seq and ERR γ ChIP-seq datasets[86]. We identified 147 Down-DRGs as ERR-activated targets (Fig. 20D and E; Table S5), which were largely associated with lipid storage (*Pparg*, *Fitm2* and *Plin3*), FAO (*Cpt2*, *Hadh*, *Hadhb*, *Acadm*, *Echs1*, *Decr1*, and *Pdk4*), the TCA cycle (*Ogdh*, *Ogdhl* and *Sucla2*), and oxidative phosphorylation (*Cox6a1*, *Ndufa13*, *Etfb* and *Coq8a*). In addition, other lipid and FAO-related genes, including *Dgat2*, *Ech1*, *Cpt1a* and *Acox1*, also showed ERRs footprints in mouse heart, but not in NMR (Fig. 20A). Notably, *Ech1*, *Dgat2*, *Nr1d2* and *Plin5* were downregulated in mouse CMs upon ERR α / γ knockdown, and *Cpt1a* has similarly been reported to be downregulated in fetal mouse CMs upon ERR α / γ knockdown. Together, these results indicate that the

strong reduction of these pathways in the NMR is mediated, at least in part, by diminished ERRs activity.

Although ERR γ is classically described as a transcriptional activator, it can also mediate transcriptional repression through interactions with corepressor complexes[164]. Consistent with this, intersection of DRGs with validated ERR-suppressed targets identified 67 Up-DRGs, suggesting a release of ERR-dependent repression in the NMR hearts (Fig. 20D and F; Table S6). Notably, these targets included genes associated with contractile function and glycogen metabolism, such as *Tnni1* and *Tpm2*, as well as *Pygl*, *Ppp1r3c*, and *Stbd1* (Fig. 15C and 20F). Interestingly, structural genes *Myh7* and *Myom3* were identified as Up-DRGs and displayed ERR footprints in NMR (Fig. 15C and 20A). Consistent with this, both genes were upregulated in mouse CMs upon ERR α/γ knockdown[86]. Surprisingly, *Pygm*, the muscle/cardiac isoform of glycogen phosphorylase, is an ERR-activated target (Fig. 20D and E; Table S5). Together with our identification of an isoform switch in the NMR heart (Fig. 16C) and the observation that *Pygm* and *Pygl* are oppositely regulated by ERR, this result suggests that reduced ERR activity may contribute to glycogen phosphorylase isoform switching in the NMR heart (Fig. 20E and F; Table S5 and S6).

Together, these findings suggest that the upregulation of neonatal-like gene programs and the retention of fetal metabolic and contractile features in the NMR heart are driven, at least in part, by reduced ERR activity. Notably, both downregulated oxidative pathways and upregulated fetal-like programs map to validated ERR-activated and -suppressed targets, respectively, indicating that these opposing transcriptional changes are coordinated through a shared regulatory axis. This supports a model in which modulation of ERR activity simultaneously diminishes activation of oxidative metabolism while relieving repression of alternative metabolic and structural programs, thereby promoting a fetal-like cardiac state. This coordinated regulation aligns with the emerging recognition that ERR is a central node linking metabolic state to contractile gene identity[83, 86].

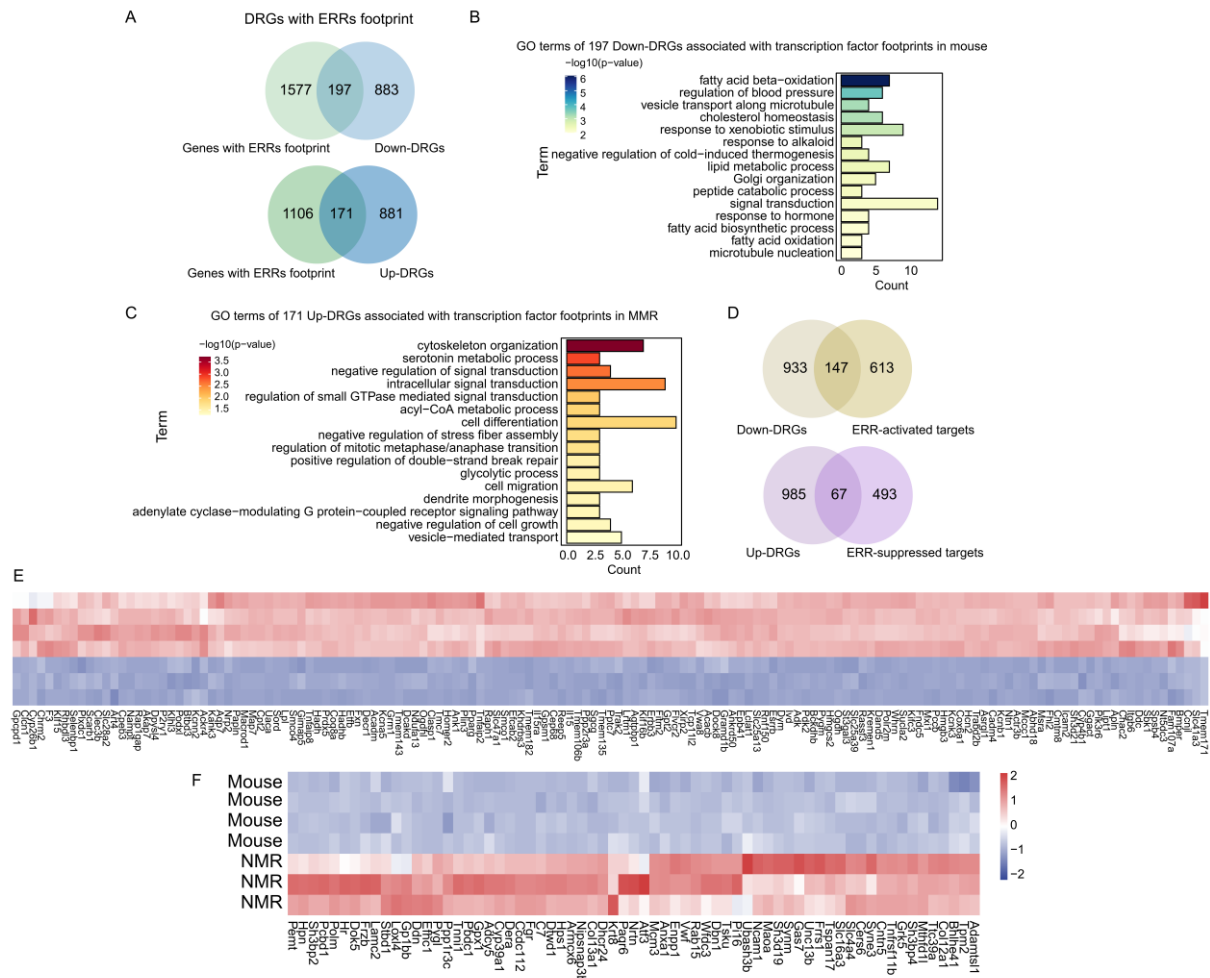


Fig. 20 Species-specific regulation of metabolic and contractile programs by ERRs in naked mole-rat and mouse hearts.

(A) Venn diagram showing the overlap between downstream target genes bound by ERRs and DRGs. (B) Gene Ontology (GO) enrichment analysis of 197 ERRs target genes overlapping with Down-DRGs. (C) GO enrichment analysis of 171 ERRs target genes overlapping with Up-DRGs. (D) Venn diagrams showing the overlap between Down-DRGs and ERR-activated targets (upper panel), and between Up-DRGs and ERR-suppressed targets (lower panel). ERR-activated and -suppressed targets were obtained from previously published datasets[86]. (E) Heatmap showing the expression of 147 ERR-activated targets within Down-DRGs. (F) Heatmap showing the expression of 67 ERR-suppressed targets within Up-DRGs.

4.2.7 ERBB2 signaling is maintained and dynamically activated in response to cardiac injury in NMR hearts

ERBB2, a co-receptor of Neuregulin-1 (NRG1), is required for NRG1-induced CM proliferation and heart regeneration through epithelial-mesenchymal transition (EMT)-like processes in mice[54, 150, 165]. Similarly, inhibition of ERBB2 in zebrafish has

been shown to impair heart regeneration[166]. In addition, many studies have highlighted a critical role of YAP in regulating CM proliferation and cardiac regeneration[89, 90, 136]. Notably, YAP activity has been reported to be regulated by ERBB2 signaling and is indispensable for ERBB2-induced cardiac phenotypes[150]. Therefore, ERBB2 functions as a central regulator of cardiac regeneration.

Erb2 expression decreases during heart maturation in mice (Fig. 21A), consistent with the progressive loss of cardiac regenerative capacity shortly after birth. Interestingly, *Erb2* was identified not only as an Up-DRG in NMR heart, but also as an up-regulated neonatal-like gene (Table S1). Although *Erb2* expression is also reduced during NMR heart development, it retains higher expression levels in adult NMR hearts than that in neonatal mouse hearts (Fig. 21B). At the epigenomic level, we observed an active promoter in adult NMR hearts, whereas in mice the same region was also marked by H3K27me3, suggesting transcriptional repression (Fig. 21C). Moreover, we identified an additional active enhancer in NMR that was completely absent in mice (Fig. 21C). Interestingly, both the promoter and this NMR-specific enhancer overlapped with NMR fast-evolving elements (Fig. 21D and E). Notably, within the promoter region, a fast-evolving element exhibited a strong bias toward W→S substitutions (Fig. 21D) ($P2 - P1 = 0.80$, p -value = 0.0026, Storer-Kim test), using a method from Liu et al[167]. Previous studies have shown that lineage-specific accelerated evolution in conserved elements is often driven by GC-biased gene conversion (gBGC) and can be associated with altered expression of nearby genes[167]. Together, these findings suggest that evolutionary changes in promoter, along with the NMR-specific activation of enhancer, may contribute to the sustained expression of *Erb2* in adult NMR hearts.

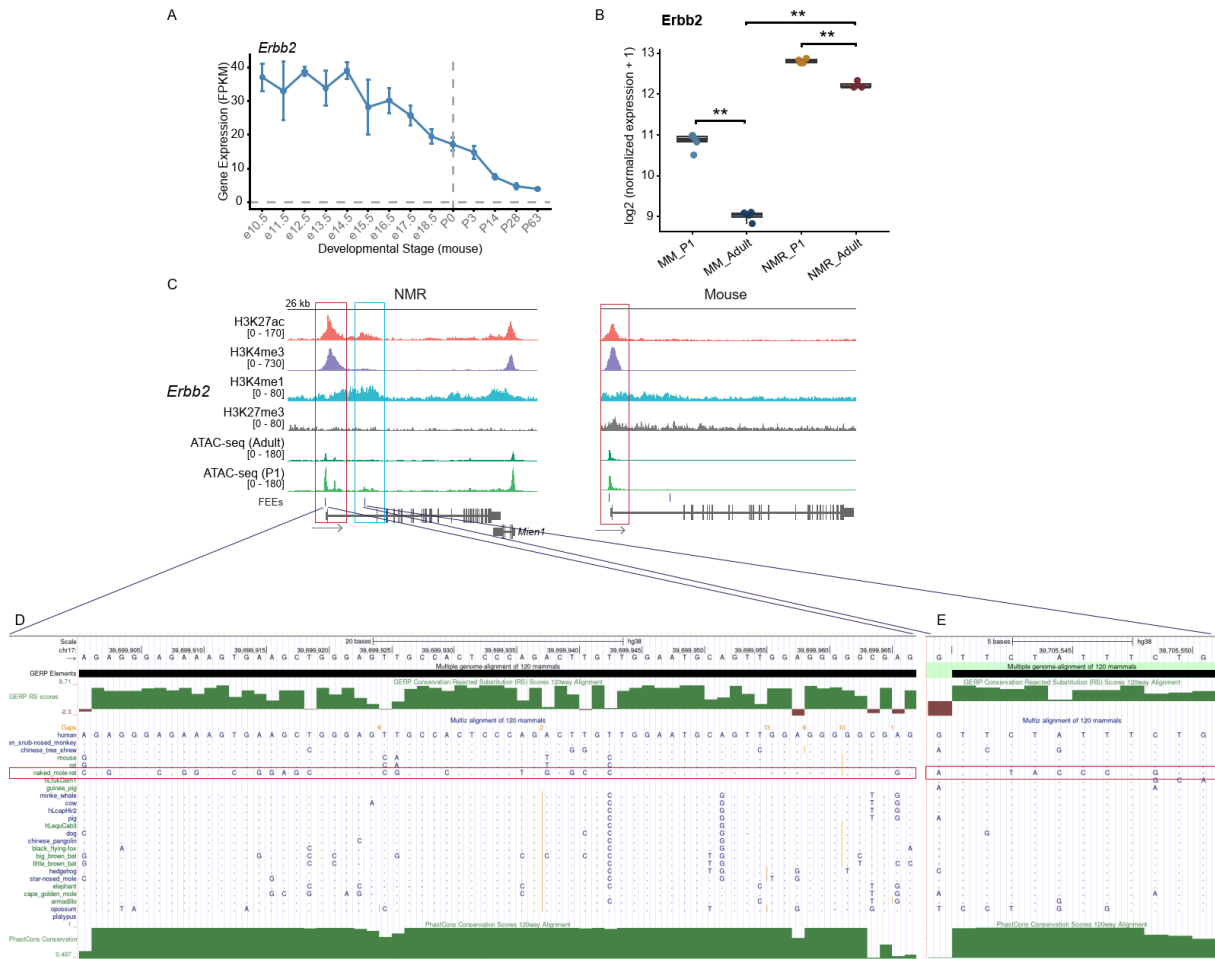


Fig. 21 Evolutionary gain of regulatory activity at the *Erbb2* locus in naked mole-rat hearts.

(A) Developmental expression dynamics of *Erbb2* during heart development in mice. (B) *Erbb2* relative expression levels in P1 and adult hearts of mouse and NMR. ** indicates adjusted p -value < 0.01 . Adjusted p -values (Benjamini-Hochberg correction) were calculated using DESeq2. (C) ChIP-seq tracks of different histone mark signals and ATAC-seq tracks at *erbb2* locus in NMR and mouse heart. Red and blue rectangles highlighted active promoter and enhancer. (D-E) Sequence alignment of two NMR rapidly evolving noncoding elements at *Erbb2* promoter (D) and enhancer (E) regions.

To further validate ERBB2 expression at the protein level, we performed Western blot analysis of heart tissues from P1 and adult mice as well as NMR samples. ERBB2 protein was detectable in neonatal mouse hearts and in both neonatal and adult NMR hearts, whereas it was completely absent in adult mouse hearts (Fig. 22A and B). Notably, ERBB2 protein levels in adult NMR hearts were significantly lower than in neonatal mouse hearts, in contrast to the corresponding transcript levels (Fig. 21B, 22A and B). This discrepancy may reflect post-transcriptional regulation or protein

turnover differences. As ERBB2 is a receptor tyrosine kinase whose activity depends on ligand-induced phosphorylation, we assessed phosphorylated ERBB2 (p-ERBB2) to evaluate its signaling status. Consistent with total ERBB2 protein levels, p-ERBB2 was detected in adult NMR hearts and was significantly higher in neonatal mouse and NMR hearts (Fig. 22A and C). These findings indicate that ERBB2 protein in adult NMR hearts is not only retained but also active under baseline conditions, suggesting a balanced activation state that may avoid excessive signaling while maintaining regenerative capacity (Fig. 22C).

To determine whether ERBB2 signaling responds to cardiac injury, we assessed total and p-ERBB2 levels in sham- and left anterior descending artery (LAD)-operated hearts at 28 days post-injury. Total ERBB2 analysis revealed sustained ERBB2 expression in NMR hearts under both sham and LAD conditions, with no significant change following injury (Fig. 22D and E). In contrast, ERBB2 protein levels in mouse hearts remained nearly undetectable in both sham and LAD conditions (Fig. 22D and E). Interestingly, p-ERBB2 was significantly increased following LAD injury (Fig. 22D and F), suggesting that CM cell cycle re-entry occurs via *ErbB2*-YAP signaling, a pathway established as critical for regeneration in zebrafish and transgenic regenerative mouse models[54, 150]. These results indicate that ERBB2 expression is maintained and primes the heart to respond to injury by inducing CM proliferation.

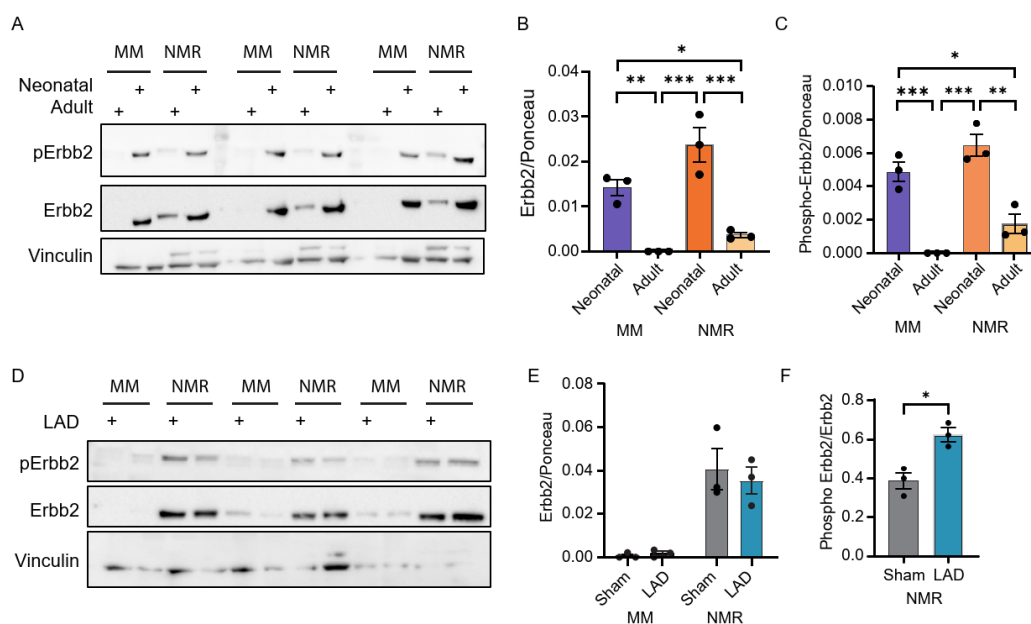


Fig. 22 Sustained ERBB2 expression and activation in adult naked mole-rat hearts under basal and injury conditions.

(A) Western blots showing phosphorylated ERBB2 (p-ERBB2), total ERBB2, and Vinculin in neonatal and adult hearts from mouse (MM) and naked mole-rat (NMR). Vinculin served as a loading control. (B) Densitometric quantification of total ERBB2 protein levels normalized to Ponceau in neonatal and adult hearts. (C) Quantification of p-ERBB2 normalized to Ponceau. (D) Western blots showing total ERBB2 and p-ERBB2 in sham- and LAD-operated hearts (28 days post-injury) from mouse and NMR. Vinculin served as a loading control. (E) Quantification of total ERBB2 normalized to total protein (Ponceau) in sham and LAD hearts. (F) Ratio of phosphorylated ERBB2 to total ERBB2 in sham- and LAD-operated NMR hearts. Each dot represents an independent biological replicate, and bars indicate mean $\pm$ SEM. Statistical significance was determined by two-way ANOVA with species and condition (or developmental stage) as independent variables. For panel F (single species, two groups), significance was assessed using an independent-samples t-test.

4.3 Hypoxia-induced transcriptional reprogramming in naked mole-rat hearts

4.3.1 Hypoxia-induced transcriptional and epigenetic reprogramming in NMR hearts towards neonatal and regenerative states

Another feature of the NMR is its exceptional adaptation to hypoxia. Previous studies have linked hypoxia to enhanced cardiac regeneration, particularly in neonatal hearts[74]. To investigate whether NMRs exhibit similar transcriptional plasticity under hypoxia, we performed RNA-seq on mouse and NMR hearts under normoxic and hypoxic conditions (Table 4). Differential expression analysis revealed a markedly greater transcriptional response in NMRs than in mice. Specifically, in NMR hearts, 468 genes were upregulated and 271 genes were downregulated under hypoxia relative to normoxia, whereas in mouse hearts, only 93 genes were upregulated and 119 genes were downregulated across conditions (Fig. 23A and B). The markedly larger number of DEGs in NMR indicates a greater transcriptional response, suggesting that NMRs maintain a robust ability to adapt to environmental changes and effectively respond to hypoxic conditions.

Table 4. Summary of RNA-seq datasets from adult mouse and NMR hearts under different oxygen conditions.

Species	Conditions	Tissue	Replicates
NMR	Normoxia	Heart	4
	Hypoxia (5%)	Heart	5
Mouse	Normoxia	Heart	4
	Hypoxia (7%)	Heart	5

To further explore the functional consequences of these transcriptional changes, we conducted GO and KEGG pathway enrichment analyses. The upregulated genes in NMR hearts under hypoxia were significantly enriched in biological processes associated with transcription regulation, cell proliferation, migration, differentiation, and development (Fig. 23C). These results suggest that hypoxia-responsive genes in NMR hearts may contribute to their enhanced regenerative capacity and remarkable adaptability to the hypoxic subterranean environment.

Additionally, we performed GSEA on the top 300 genes highly expressed in mouse neonatal CM compared to adults under different oxygen conditions in the NMR database. We found that these neonatal CM genes were significantly enriched among the highly expressed genes in NMR hearts under hypoxia (Fig. 23D). We also found that among the genes upregulated under hypoxia in the NMR, 38 genes were significantly downregulated in mouse hearts during maturation (Fig. 23E). These included metabolic regulators such as *Ppp1r3g*, a glycogen-related gene, and *Slc16a3*, a monocarboxylate transporter that promotes a metabolic shift towards glycolysis.

In addition to metabolic regulators, several key transcription factors (*Pitx2*, *Hey2*, *Sfrp1*, *Wt1*, *Tgif2*, *Myc* and *Id2*) associated with cardiac development and regeneration were activated in NMR hearts under hypoxia[168–172] (Fig. 23E and F). Notably, *Pitx2* is essential for neonatal heart regeneration, and its overexpression promotes cardiac repair in adult CMs after injury by activating antioxidant responses[169]. Interestingly, in both adult NMR and mouse hearts, the *Pitx2* gene body is broadly covered by the repressive histone mark H3K27me3, whereas an open chromatin signal emerges in P1 hearts (Fig. 23G). Similarly, *Hey2*, *Sfrp1*, *Wt1*, and *Tgif2* all exhibited bivalent status in hearts under normoxia (Fig. 23H). Additionally, *Pitx2*, *Hey2* and *Wt1* all have NMR fast-evolving elements. This suggests that epigenetic regulation may underlie the changes in gene expression, and that hypoxia in NMR hearts could modulate these chromatin states to reactivate developmental and regenerative transcriptional programs.

Furthermore, genes associated with heart regeneration as reported in previous studies were also enriched among the hypoxia-upregulated genes in NMR (Fig. 23I). Among the core enrichment genes, *Fosb*, *Fosl1* and *Fosl2*, members of the Fos gene family, were identified (Fig. 23I). The Fos gene family consists of 4 members: c-FOS, FOSB, FOSL1, and FOSL2, which form the activator protein 1 (AP-1) transcription factor

complex with Jun proteins. It plays an essential role in CM proliferation and heart regeneration in vertebrates[60, 88]. Remarkably, *Fosl1* and *Fosb* were exclusively expressed in hypoxic NMR hearts, but not in normoxia, nor in mouse hearts under either oxygen condition (Fig. 23J). Both genes displayed bivalent promoters and poised enhancers in normoxia in both species (Fig. 23K), further supporting the notion that hypoxia unlocks epigenetically restrained regenerative programs.

Together, these results indicate that hypoxia shifts the transcriptional landscape of NMR hearts toward a more neonatal-like and regeneration-associated state, highlighting the remarkable transcriptional plasticity of the NMR myocardium and its capacity to mount a regenerative response under hypoxic stress.

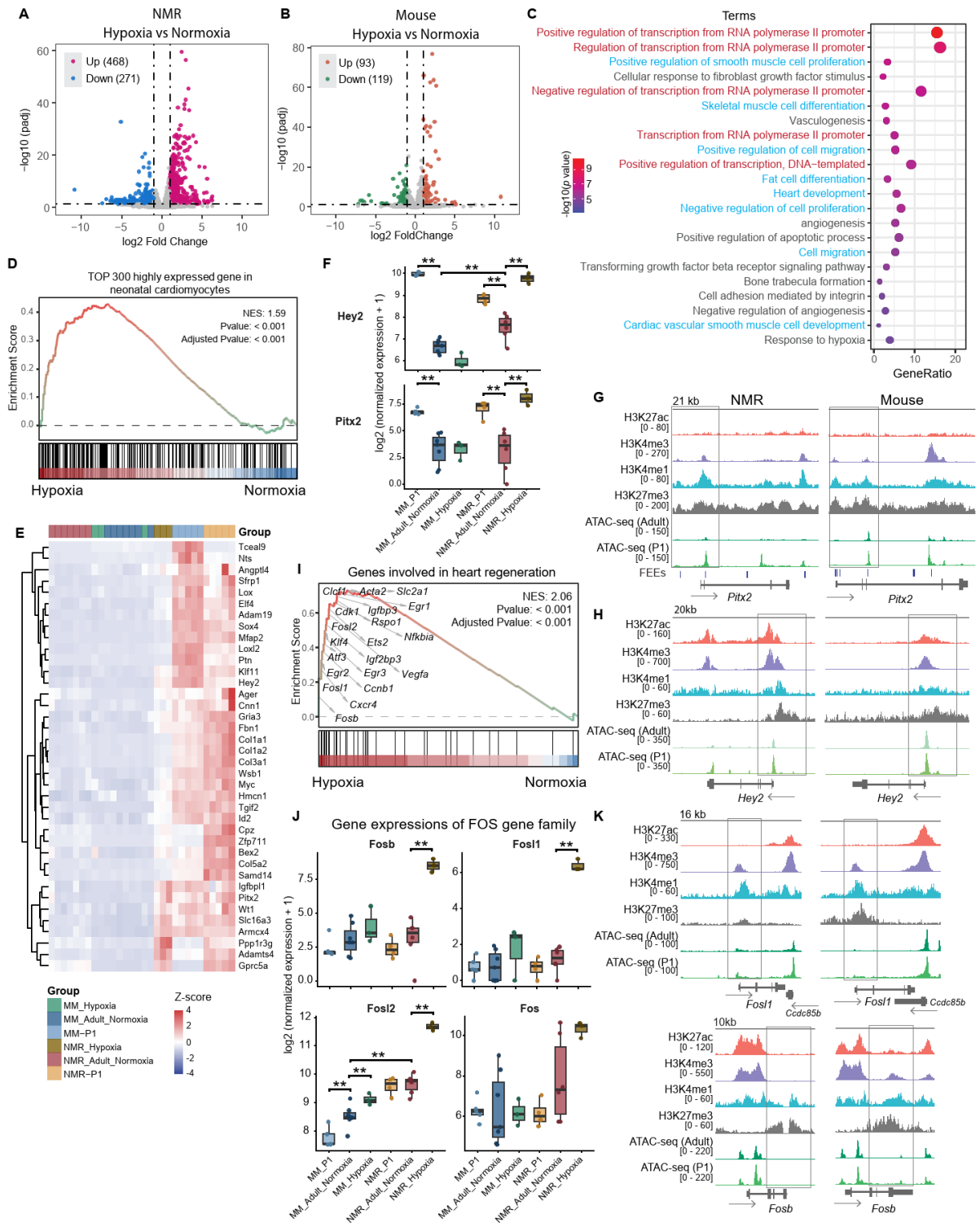


Fig. 23 Hypoxia reactivates developmental and regenerative transcriptional programs through epigenetic remodeling in NMR hearts.

(A) Volcano plot showing DEGs in NMR hearts under hypoxia compared with normoxia. Pink and blue dots represent hypoxia-upregulated and -downregulated genes, respectively. Dashed lines indicate thresholds of $|\text{Log}_2(\text{fold change})| = 1.5$ and $-\log_{10}(\text{adjusted } p) = 2$. (B) Volcano plot showing DEGs in mouse hearts under hypoxia compared with normoxia. Green and orange dots represent hypoxia-

upregulated and -downregulated genes, respectively. (C) Dot plot of GO enrichment analysis for hypoxia-upregulated genes in NMR hearts. Terms related to transcriptional regulation are highlighted in red, whereas terms associated with cell development, proliferation, and differentiation are highlighted in blue. (D) Gene set enrichment analysis (GSEA) showing that TOP 300 genes highly expressed in neonatal mouse cardiomyocytes (P1 vs. adult) are significantly enriched among hypoxia-upregulated genes in NMR hearts. (E) Heatmap showing expression patterns of 38 genes upregulated in hypoxic NMR hearts (vs. normoxia) and also upregulated in P1 mouse hearts (vs. adult). (F) Box plots showing gene relative expression of *Hey2* and *Pitx2* in NMR and mouse hearts under different oxygen conditions and developmental stages. ** indicate adjusted p-value < 0.01. Adjusted p-values (Benjamini-Hochberg correction) were calculated using DESeq2. Adult samples from the developmental dataset and the hypoxia control dataset were combined as the Adult_Normoxia group. (G and H) IGV browser tracks showing various chromatin features at the genomic region around *Pitx2* (G) and *Hey2* (H) in NMR and mouse. (I) GSEA result of heart regeneration related genes are significantly enriched among hypoxia-upregulated genes in NMR hearts. (J) Box plots showing relative expression patterns of *Fos* gene family members (*Fosb*, *Fosl1*, *Fosl2* and *Fos*) in NMR and mouse hearts under different oxygen conditions and developmental stages. ** indicate adjusted p-value < 0.01. Adjusted p-values (Benjamini-Hochberg correction) were calculated using DESeq2. Adult samples from the developmental dataset and the hypoxia control dataset were combined as the Adult_Normoxia group. (K) IGV browser tracks showing various chromatin features at *Fosl1* and *Fosb*. The bivalent regions are highlighted by rectangular boxes.

4.3.2 Two classes of bivalent promoters define divergent chromatin states and gene regulatory functions in Hearts

Previous results have shown that NMR hearts exhibit broader H3K27me3 coverage compared to mouse hearts, and that some bivalent promoters can switch to an active state under stress, thereby promoting gene expression programs related to environmental adaptation (Fig. 13A; Fig. 23F-K). To further investigate the impact of H3K27me3 on the transcriptional profile of NMR hearts, and to comprehensively identify and compare genes regulated by bivalent elements between NMR and mouse hearts, we next explored the regulatory functions of H3K27me3 in both species.

We first annotated the genomic distribution of H3K27me3 consensus peaks in both species and found that they were predominantly located at promoter and intergenic regions in both NMR and mouse hearts (Fig. 24A). We next examined the distribution of other histone modifications across all H3K27me3 consensus peaks. The results showed that the majority of these regions were marked exclusively by H3K27me3, accounting for 66% in NMR and 51% in mouse hearts (Fig. 24B). The remaining peaks were primarily co-occupied by all three other histone modifications (10-18%) or marked by H3K4me3 and H3K4me1 (11-14%), and these two peak sets also displayed the

strongest H3K27me3 signal intensity (Fig. 23G, H and K; Fig. 24B and C). Consistent with our findings, previous studies have demonstrated that most classical bivalent promoters are in fact “trivalent,” as they carry H3K4me1 in addition to the canonical H3K4me3 and H3K27me3 modifications[173, 174]. Moreover, the co-occurrence of H3K27ac at H3K27me3-marked loci suggests that these regions may represent a transitional chromatin state from repression to activation, or reflect cellular heterogeneity. Notably, bivalent promoters exhibited two distinct chromatin states: Type I, co-marked with H3K27ac and characterized by stronger H3K4me3 and H3K27ac signals than Type II; and Type II, lacking H3K27ac and showing stronger enrichment of H3K27me3 than Type I (Fig. 24C).

To systematically investigate these two types of loci, we performed comparative analyses of Type I and Type II bivalent promoters to assess their distinct chromatin features and potential regulatory functions. In total, we detected 2,691 Type I and 3,800 Type II bivalent promoters in NMR hearts, and 3,380 Type I and 3,506 Type II bivalent promoters mouse hearts (Fig. 24D). Among them, 73.27-86.75% of Type I elements and 51.21-67.97% of Type II elements were located within 2 kb of annotated transcription start sites (TSSs) (Fig. 24D), suggesting that Type I promoters are more strongly associated with proximal promoter regions. Furthermore, compared with genes marked by Type I promoters, genes associated with Type II bivalent promoters showed significantly lower expression levels in both species (Fig. 24E). Consistently, GO enrichment analysis showed that while both gene sets were enriched in terms related to transcriptional regulation processes, Type I genes were preferentially associated with cell proliferation and cardiac development, whereas Type II genes were predominantly enriched in cell differentiation and neural-related pathways (Fig. 24F and G). Together, these results indicate that Type I and Type II bivalent promoters reveal conserved yet distinct regulatory influences in the heart, raising the possibility that both of their dynamic modulation may contribute to transcriptional reprogramming under stress conditions.

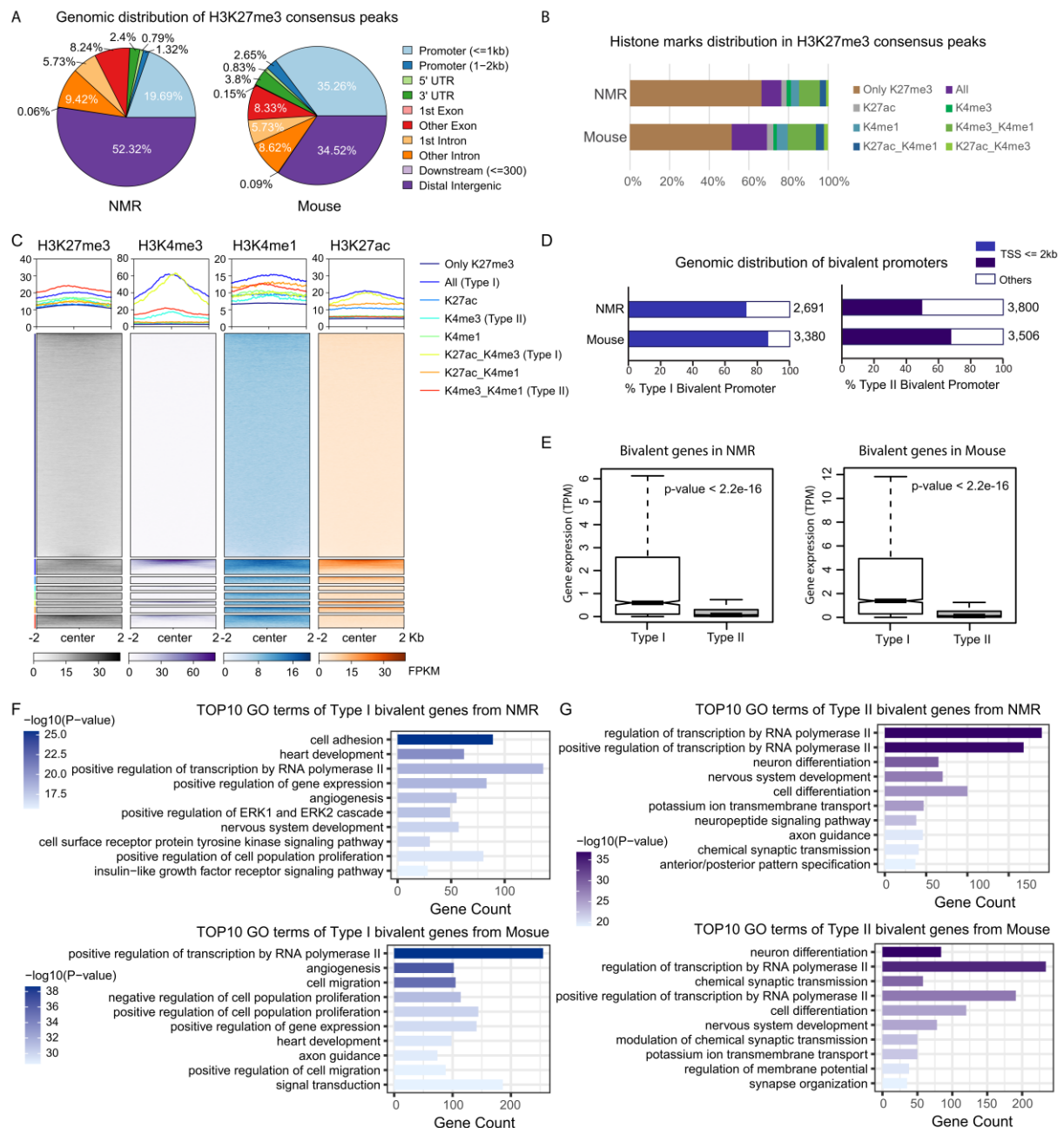


Fig. 24 Distinct chromatin states and functional features of bivalent promoters in NMR and mouse hearts.

(A) Pie charts showing the genomic distribution of H3K27me3 consensus peaks in NMR (left) and mouse (right) hearts. (B) Bar plots comparing the relative proportions of other histone marks (H3K4me3, H3K4me1, and H3K27ac) across H3K27me3 consensus peaks in NMR and mouse hearts. (C) Average plots and heatmaps showing the signals from different peak sets at the peak center in ChIP-seq data. (D) Bar plots showing the genomic distribution of Type I (left) and Type II (right) bivalent promoters in NMR and mouse hearts. The colored bar indicates promoters located within 2 kb of annotated transcription start sites ($TSS \leq 2$ kb). (E) Expression levels of genes associated with Type I and Type II bivalent promoters in NMR (left) and mouse (right). Bar plots showing TOP10 GO terms for Type I (F) and Type II (G) bivalent genes in NMR and mouse hearts.

4.3.3 Hypoxia-induced reactivation of bivalent genes supports ECM remodeling and stress adaptation in NMR hearts

To assess whether bivalent genes contribute to the remarkable environmental adaptability of NMRs, we integrated heart transcriptome data from hypoxic conditions. Among all NMR bivalent genes, we identified 104 genes that were significantly upregulated under hypoxia (Fig. 25A). Notably, 80 of these genes were also classified as bivalent in mouse hearts. However, only one gene was upregulated in mouse hearts under hypoxic conditions. These results indicate that bivalent genes in NMR hearts exhibit a species-specific transcriptional activation in response to hypoxia, highlighting their potential role in hypoxia tolerance. GO enrichment analysis of the 104 hypoxia-upregulated NMR bivalent genes revealed a strong enrichment in processes related to cardiac and vascular development, including heart development, vasculogenesis, blood vessel development, branching morphogenesis of blood vessels, and angiogenesis (Fig. 25B). In addition, terms associated with extracellular matrix (ECM) organization, integrin-mediated cell adhesion, and cell migration were significantly enriched, reflecting pathways essential for tissue remodeling and adaptive responses under hypoxia (Fig. 25B). Moreover, pathways such as response to cAMP and platelet-derived growth factor receptor-beta signaling highlight signaling events relevant to stress responses (Fig. 25B).

Among these genes, there are 17 genes upregulated in P1 mouse compared to adult mouse hearts. In addition to *Pitx2*, *Hey2*, *Wt1*, *Sfrp1* and *Ppp1r3g* we mentioned before, we also found *Lox/2* and *Lox*, encoding lysyl oxidases that catalyze collagen crosslinking. Both genes have been implicated in ECM remodeling, myocardial fibrosis, and cardiac stress responses, highlighting their role in maintaining ECM stiffness and structural adaptation under pathological or hypoxic conditions[175, 176]. *Fbn1*, encoding fibrillin-1, is a critical structural glycoprotein in the microfibrillar network, and its dysregulation has been implicated in impaired elastic fiber assembly and pathological ECM remodeling in cardiac disease[177]. Furthermore, *Cpz*, a secreted carboxypeptidase, has been reported as an ECM component in certain cell types and functions as a modulator of Wnt signaling, thereby linking ECM composition with developmental and stress-responsive pathways[178, 179]. In addition, *Igfbp1*, encoding insulin-like growth factor binding protein-like 1, was also identified among these hypoxia-responsive bivalent genes in NMR (Fig. 25C and D). Insulin-like growth

factors (IGFs) are key modulators of cardiac development and CM proliferation across vertebrates[180]. Importantly, the transcriptional silencing of *Igfbp1* in various cell types has been shown to be mediated by promoter DNA methylation, highlighting its epigenetic regulation[181]. Beyond individual genes, GSEA of hallmark pathways revealed that epithelial-mesenchymal transition was significantly enriched among hypoxia-upregulated genes in NMR hearts, consistent with the activation of transcriptional programs associated with ECM remodeling and cellular plasticity (Fig. 25E). Collectively, these findings suggest that hypoxia-induced reactivation of ECM- and signaling-related bivalent genes contributes to enhanced transcriptional flexibility, thereby facilitating adaptive tissue remodeling and stress resilience in NMR hearts under low-oxygen conditions.

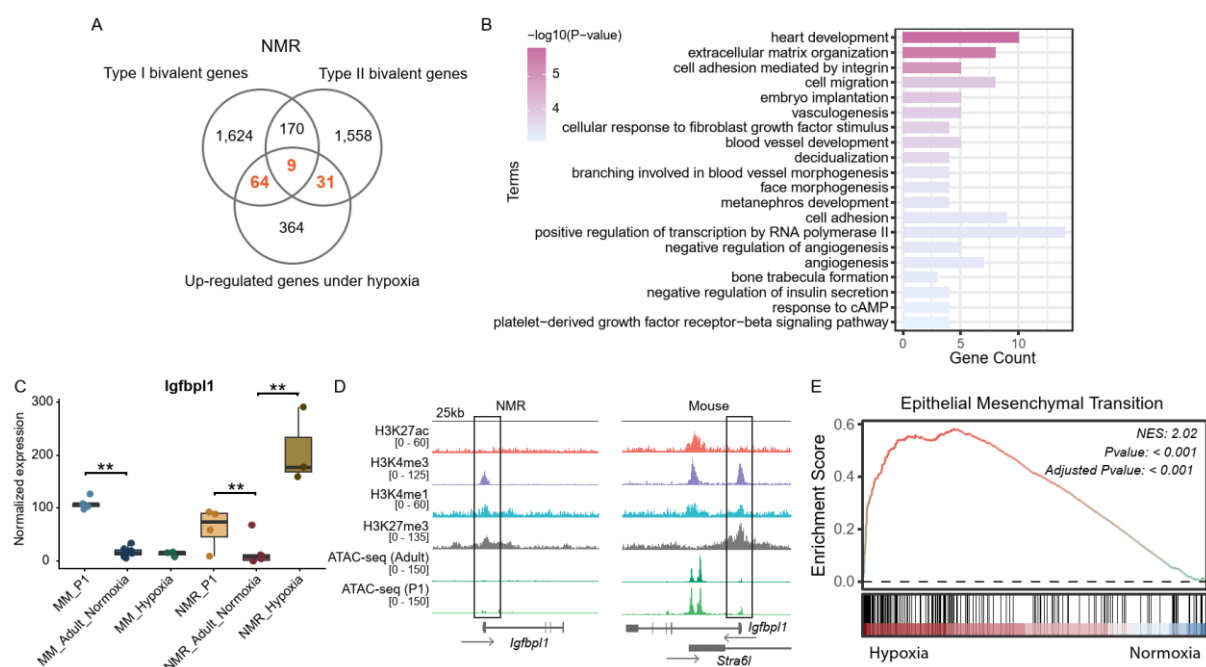


Fig. 25 Hypoxia-induced activation of bivalent genes in NMR hearts.

(A) Venn diagram shows overview of the integration of bivalent gene annotation with hypoxia-responsive RNA-seq data from NMR. A total of 104 NMR bivalent genes were significantly upregulated under hypoxia. (B) GO enrichment result of the 104 hypoxia-upregulated NMR bivalent genes. (C) Box plots showing relative gene expression levels of *Igfbp1* across different oxygen conditions and developmental stages. ** indicate adjusted p -value < 0.01 . Adjusted p -values (Benjamini-Hochberg correction) were calculated using DESeq2. Adult samples from the developmental dataset and the hypoxia control dataset were combined as the Adult_Normoxia group. (D) IGV browser tracks displaying various chromatin features at the genomic region around *Igfbp1*. The bivalent promoter regions are highlighted by black rectangular boxes. (E) GSEA showing that Epithelial Mesenchymal Transition genes are significantly enriched among hypoxia-upregulated genes in NMR hearts.

5 Discussion

Across the animal kingdom, the capacity for organ regeneration varies substantially and generally declines with increasing organismal complexity[182]. Invertebrates exhibit remarkable regenerative abilities, enabling the reconstruction of entire body structures following injury. For example, organisms such as hydra and planaria can regenerate a complete body from small tissue fragments after amputation[183–186]. Among vertebrates, several lower vertebrate species also retain extensive regenerative potential throughout life. Zebrafish and salamanders, for instance, can regenerate a wide range of complex tissues and organs, including the heart, fins/limbs and spinal cord[39, 187–189].

In contrast, regenerative capacity in mammals is markedly restricted. Only a limited number of mammalian tissues display robust regenerative potential, and complete organ regeneration is extremely rare. Deer antlers are the only known mammalian appendage capable of full and repeated regeneration[190, 191]. In addition, some mammals, such as rabbits and African spiny mice, can regenerate parts of their outer ear following injury[192]. With respect to the heart, mammals exhibit only a transient regenerative window during the early neonatal period. In mice, for example, the heart retains regenerative capacity for approximately the first 7 days after birth, after which CMs largely exit the cell cycle and the heart undergoes rapid postnatal maturation[41, 47]. Recent studies have shown that this transition is accompanied by metabolic reprogramming, cell cycle withdrawal, cytoskeletal and cytoarchitectural remodeling, and large-scale transcriptional changes[68, 193–195]. Despite intensive investigation, the molecular mechanisms underlying rapid decline of cardiac regenerative capacity during postnatal maturation remain incompletely understood.

The NMR provides a unique model for exploring these questions. As an exceptionally long-lived subterranean rodent, the adult NMR heart exhibits remarkable physiological adaptations, including hypoxia tolerance, neonatal-like metabolic characteristics, resistance to ischemic injury, and the capacity to regenerate myocardium following cardiac injury[10, 93]. However, the genetic and molecular mechanisms underlying these distinctive cardiac features remain largely unexplored.

In this thesis, this question was addressed through a comparative multi-omics analysis of mouse and NMR hearts across developmental stages and oxygen conditions. Our

results demonstrate that adult NMR hearts retain neonatal-like gene expression, including the persistent expression of genes associated with cell cycle regulation and neonatal sarcomere organization. Furthermore, DRGs, particularly those involved in cytoskeletal organization and metabolic switching, appear to be modulated by cis-regulatory elements, suggesting that remodeling of the regulatory landscape contributes to the distinctive cardiac phenotype observed in adult NMRs. Notably, the transcription factor *Esrrg*, a key regulator of metabolic remodeling, appears to play a central role in shaping the metabolic program of the NMR heart. In addition, our heart injury samples revealed increased phosphorylation-dependent activation of ERBB2 in adult NMR hearts following cardiac injury, whereas this activation was not observed in mice, suggesting that ERBB2 signaling may contribute to the regenerative response in this species. In addition, genes associated with bivalent regulatory elements were activated under low oxygen conditions, which may facilitate rapid transcriptional responses to environmental stress and contribute to the remarkable stress resistance observed in NMRs.

5.1 Persistent neonatal-like gene expression in the adult naked mole-rat heart

Consistent with developmental transitions during heart maturation, our PCA results revealed that both NMR and mouse hearts undergo major transcriptomic changes during postnatal development (Fig. 10A). However, in addition to these shared developmental changes, adult NMR hearts retain aspects of neonatal-like gene expression patterns. In particular, genes that exhibit significant developmental regulation in mice remain relatively unchanged in adult NMR hearts, indicating that elements of the neonatal transcriptional program in mice persist beyond early development in NMRs.

Several studies have suggested that the loss of regenerative capacity during cardiac maturation is closely associated with the suppression of fetal or neonatal gene expression programs[41]. Reactivation of these developmental gene networks has been shown to partially restore proliferative potential in adult CMs and to promote cardiac repair following injury[54, 196, 197]. In the present study, we identified 466 neonatal-like genes that remain in adult NMR hearts. These genes are associated with cell cycle, cytoskeletal organization, early cardiac development, and metabolic remodeling. In addition, CM proliferation was still detectable in NMR hearts one month

after birth, whereas such proliferative activity was not observed at the adult stage. This prolonged postnatal proliferative activity may also be related, at least in part, to the higher proportion of mononucleated CMs observed in NMR hearts, as mononucleated CMs are generally considered more permissive for cell cycle re-entry than multinucleated CMs[49]. Together, these findings suggest that the transcriptional landscape of the NMR heart does not fully transition to the canonical adult state observed in other mammals. Instead, neonatal-like gene expression persists into adulthood, which may contribute to the maintenance of neonatal-like cardiac features in NMRs and potentially underlie their capacity for cardiac regeneration.

Interestingly, reactivation of certain fetal genes is also a well-recognized feature of cardiac stress and disease in mammals. Under pathological conditions such as cardiac hypertrophy or heart failure, adult CMs often re-express fetal gene programs as part of an adaptive response to stress[198–201]. However, in most mammalian species, this reactivation is typically associated with pathological remodeling rather than functional regeneration[199, 202]. In contrast, the persistent expression of neonatal-like genes observed in NMR hearts represents a fundamentally different biological context, in which developmental gene programs are retained as part of the normal physiological state rather than being reactivated only under pathological conditions. Consequently, the neonatal-like gene set identified in this study provides valuable candidate regulators that may contribute to the maintenance of immature cardiac features and potentially support regenerative competence in adult mammalian hearts.

It should be noted, however, that the neonatal-like genes identified in this study cannot be unequivocally attributed to CMs. Because the transcriptomic data were derived from whole-heart tissues, observed gene expression patterns may also reflect contributions from other cardiac cell populations, including fibroblasts, endothelial cells, and immune cells[203–205]. Although several of the identified genes have previously been reported to be expressed in CMs and to promote cardiac regeneration following injury, the precise cellular origin of many neonatal-like genes remains unclear[206]. Therefore, to determine the cell-type-specific sources of these neonatal-like genes, future studies using single-cell transcriptomics or spatially resolved transcriptomic approaches will be required[207, 208]. Such approaches will help to further dissect the cell-type-specific expression patterns of these genes and clarify whether they directly contribute to CM proliferation and regeneration in NMR hearts.

5.2 Cytoskeletal remodeling and neonatal sarcomeric isoforms contribute to a regenerative cardiac state

Previous studies have shown that sarcomeric disassembly is a critical feature of heart regeneration in adult zebrafish and neonatal mice[41, 44]. In contrast, CMs in non-regenerative adult mammalian hearts have highly organized, well-aligned sarcomeres, and exhibit tightly packed intercellular spaces. Consistent with this distinction, our previous findings suggest that CMs in the adult NMR heart exhibit a less organized structure with enlarged intercellular spaces, resembling a neonatal-like phenotype. In the present study, cytoskeleton-related pathways were significantly enriched among up-DRGs in adult NMR hearts, as well as among genes associated with NMR-specific fast-evolving elements (Fig. 15A and Table 3). Notably, many of these genes are involved in actin filament organization, including *Spire1/2*, *Cobl*, *Was*, *Phactr1*, *Phldb2*, *Dbn1*, and *Eps8*, as well as in sarcomeric structure, such as *Tnni1*, *Myh7*, *Tnnt1*, and *Tpm2*. These observations suggest that cytoskeleton-associated genes may be subject to species-specific cis-regulatory control in NMRs, thereby contributing to the structural remodeling.

Furthermore, motif enrichment analysis of NMR-specific more-active accessible enhancers revealed a strong enrichment of AP-1 binding motifs (Fig. 18D). AP-1 has been shown to promote sarcomere disassembly during zebrafish heart regeneration, and its downstream target gene *Fbxl22* is also an UP-DRG in adult NMR heart. Notably, overexpression of *Fbxl22* can further promote sarcomere disassembly[60], suggesting that AP-1-dependent transcriptional programs may contribute to structural remodeling in the NMR myocardium. In addition, we observed isoform switches in sarcomeric proteins, with the neonatal isoform *Tnni1* and *Myh7* being UP-DRGs identified in adult NMR hearts, while the adult isoforms *Tnni3* and *Myh6* were relatively reduced. This isoform shift may be linked to altered ERR signaling, as *Tnni1* and *Myh7* has been reported as ERR-suppressed targets, whereas *Tnni3* is an ERR-activated target[86]. Accordingly, the reduced expression of *Esrrg* and *Esrrb* in NMR hearts may contribute to this isoform transition. However, the regulation of the *Myh7-Myh6* locus is likely more complex. In addition to ERR signaling, these genes are associated with ERRγ-containing super-enhancers, suggesting that their expression may be co-regulated by additional transcription factors[164].

The structural organization of CM is closely linked to their proliferative capacity. During postnatal maturation, the establishment of highly ordered sarcomeres and a stabilized cytoskeletal network imposes significant physical constraints on cell division[41, 44]. Successful mitosis requires dynamic cytoskeletal rearrangement, including sarcomere disassembly and the formation of mitotic structures such as the spindle apparatus and contractile ring[209, 210]. In mature CMs, however, densely packed myofibrils and rigid cytoskeletal architecture can hinder these processes, thereby limiting cell cycle re-entry. In contrast, the cytoskeletal remodeling observed in adult NMR hearts may create a structural environment that is more permissive for cell cycle re-entry. Our results show that cytoskeleton-related genes are significantly up-regulated in NMR hearts, suggesting enhanced regulation of actin dynamics and structural plasticity. Consistent with this idea, actin nucleation factors such as *Spire1/2* have been implicated in regulating cytokinesis, as disruption of their function impairs actin organization and leads to failure of cell division[211]. These findings support the notion that actin cytoskeleton remodeling is a critical prerequisite for successful cell division.

In addition to structural remodeling, metabolic rewiring in NMR hearts may further support cell cycle re-entry by facilitating membrane biosynthesis[212]. Our data indicate that fatty acids are redirected away from oxidation or storage and instead preferentially utilized for the synthesis of glycerophospholipids, including phosphatidylethanolamine (PE) and phosphatidylcholine (PC) (Fig. 15A; 17C, F and G). As these lipids are essential components of cellular membranes, their increased availability may support membrane expansion and cytokinesis during cell division[213]. Together, these findings suggest that coordinated regulation of cytoskeletal dynamics and lipid metabolism may establish a permissive cellular environment that supports regeneration in the NMR heart.

Furthermore, cytoskeletal organization is not only a structural feature but also a dynamic regulator of signaling. Recent studies have shown that myosin-generated forces can remodel F-actin, exposing binding sites for mechanosensitive proteins and thereby facilitating signal transduction[214]. In this context, the cytoskeletal architecture remodeling observed in NMR hearts may enhance the recruitment and activation of signaling pathways involved in CM proliferation and regeneration. Among these, ERBB2 signaling plays a key role in heart regeneration, and constitutively active *ErbB2* (*caErbB2*) in adult mouse hearts has been shown to induce cytoskeletal

remodeling via YAP activation during heart regeneration[54, 150]. Notably, our results indicate that *ErbB2* is not only an upregulated neonatal-like gene in adult NMR hearts, associated with NMR-specific fast-evolving regulatory elements, but is also detectable at the protein level and functionally active (Fig. 22A-C). However, both total ERBB2 protein abundance and its phosphorylation levels are much lower than those observed in neonatal hearts, suggesting that ERBB2 signaling may be finely tuned rather than fully reactivated. Given that overexpression of *caErbB2* has been shown to induce cardiomegaly[54], the moderate activation of ERBB2 observed in adult NMR heart may represent a protective regulatory strategy. This controlled activation may allow the engagement of regenerative signaling when required, while limiting excessive CM proliferation and potential pathological remodeling.

Taken together, these findings support a model in which cytoskeletal remodeling, neonatal sarcomeric isoform expression, metabolic reprogramming, and finely tuned regenerative signaling collectively establish a structurally and molecularly permissive state in the NMR heart, enabling cardiomyocytes to retain regenerative potential without undergoing uncontrolled proliferation.

5.3 ERR-driven metabolic reprogramming in the naked mole-rat heart

Estrogen-related receptors (ERRs) have been shown to play essential roles in postnatal cardiac maturation by driving the expression of genes involved in mitochondrial energy metabolism[86]. In mice, ERRs are upregulated during heart maturation and are required for the transition toward oxidative metabolism. Consistent with this, our motif enrichment analysis of differential H3K27ac peaks integrated with chromatin accessibility data revealed that ERR β and ERR γ binding motifs are significantly enriched in mouse more-active accessible enhancers, whereas such enrichment is largely absent in NMR hearts (Fig. 18E), also accompanied by reduced ERR expression in NMR (Fig. 19A).

To further define ERR-dependent regulatory networks, we performed DNA footprinting analysis in both mouse and NMR hearts, thereby enabling inference of species-specific ERR binding events. By integrating these results with previously identified ERR target genes and our DRGs, we found that ERR acts as a central transcriptional regulator promoting metabolic maturation in the mouse heart. In contrast, reduced ERR activity in NMR hearts is associated with Down-DRGs involved in FAO, TCA cycle, oxidative

phosphorylation, and lipid storage. Conversely, several Up-DRGs related to glycogen metabolism, including *Pygl*, *Ppp1r3c*, and *Stbd1*, were identified as ERR-suppressed targets. Notably, we also observed an isoform switch of glycogen phosphorylase from the muscle to the liver type in NMR hearts, consistent with reduced ERR activity. Together, these findings indicate that ERR functions as a key regulator of cardiac metabolic remodeling, driving the transition toward oxidative metabolism during postnatal maturation. The attenuation of ERR signaling in NMR hearts may therefore contribute to the maintenance of a neonatal-like metabolic state, potentially helping to overcome metabolic barriers to cardiac regeneration.

Interestingly, we also observed that peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 α), a well-established coactivator of nuclear receptors including ERRs[84], is significantly downregulated in NMR hearts (Fig. 17E). This suggests that coordinated changes in both ERRs and their coactivators may contribute to the reprogramming of the cardiac metabolic regulatory landscape. Furthermore, NMR-specific amino acid substitutions in ERR proteins, together with the presence of NMR-specific fast-evolving elements, may further contribute to species-specific modulation of ERR activity.

Importantly, our data suggest that suppression of ERR signaling in NMR hearts does not simply inhibit fatty acid utilization. We observed that NMR hearts exhibit reduced FAO without accumulating lipid droplets (Fig. 17C), accompanied by downregulation of triglyceride biosynthesis, utilization, and lipid homeostasis pathways (Fig. 15A). Rather than being stored, fatty acids appear to be preferentially utilized for glycerophospholipid biosynthesis (Fig. 15A). In contrast, ERR α/γ knockdown in mouse hearts leads to lipid droplet accumulation, indicating that loss of ERR suppresses FAO but does not necessarily promote alternative fatty acid utilization pathways[86]. Consistent with this, an integrated analysis of ERR γ knockdown H3K27ac profiles and ERR γ ChIP-seq data suggests that mitochondrial energy metabolism and classical ERR-activated targets are primarily regulated by ERR γ alone, whereas other target genes may be co-regulated by additional transcription factors through ERR-containing super-enhancers (SEs)[215]. For example, *Gpat3*, a key rate-limiting enzyme in glycerophospholipid biosynthesis, is an Up-DRG in NMR hearts and an ERR target associated with ERR-containing SEs[215]. Notably, other Up-DRGs involved in

phospholipid metabolism are not direct ERR targets, suggesting that additional regulatory mechanisms contribute to this fatty acid redirection.

Furthermore, emerging evidence suggests that metabolic remodeling may be linked to YAP signaling. Overexpression of constitutively active YAP (YAP5SA) in adult mouse hearts promotes the establishment of a neonatal-like niche and enhances cardiac regeneration[89, 196]. Notably, TEAD-dependent YAP activation has also been reported to reduce triglyceride levels and promote glycerophospholipid synthesis[216]. In this context, the metabolic phenotype observed in NMR hearts may also involve YAP-TEAD signaling. Although *Yap1* expression is relatively low in adult NMR hearts, *Tead2* and *Tead4* are significantly upregulated compared with mice, suggesting that differential TEAD activity may play a dominant role in driving downstream programs. For instance, *Pik3cb*, an enzyme linking Hippo-YAP and PI3K-AKT signaling pathways through direct TEAD binding and promoting CM proliferation[217], is an up-DRG in NMR hearts (Fig. 17G). As phosphatidylinositol, a key substrate of PIK3CB, belongs to the glycerophospholipid class, the increased glycerophospholipid biosynthesis observed in NMR hearts may further influence downstream signaling pathways. Importantly, genes associated with NMR fast-evolving elements are also functionally enriched in the PI3K-Akt signaling pathway (Table 3), suggesting that regulatory evolution may also have reshaped phosphoinositide-mediated signaling in NMR hearts. Taken together, these findings identify ERR as a central regulator of metabolic remodeling in the NMR heart and suggest that additional signaling pathways may cooperate to shape a metabolic state that potentially supports regeneration.

5.4 Stress-responsive signaling promotes regenerative plasticity in NMR hearts
Following cardiac injury and environmental stress, the heart can undergo either regeneration or pathological remodeling, depending on the ability of CMs to activate appropriate stress-responsive programs[41, 218]. In adult mammals, stress responses are insufficient to induce heart regeneration and instead lead to fibrosis and loss of function. In contrast, regenerative vertebrates and neonatal mammals retain the ability to activate pro-regenerative programs in response to stress.

Our results indicate that ERBB2 signaling is selectively activated in adult NMR hearts following cardiac injury, as evidenced by increased phosphorylation levels despite no significant change in total ERBB2 protein levels (Fig. 22D and F). Given the established

role of ERBB2 in promoting CM dedifferentiation, metabolic reprogramming, cytoskeletal remodeling, and cell cycle re-entry[54, 70, 150], this finding suggests that NMR hearts retain the ability to engage key pro-regenerative signaling pathways in response to injury.

In addition to injury-induced signaling, hypoxia is a physiologically relevant stress condition for NMRs, which naturally inhabit low-oxygen subterranean environments[10]. Our results show that hypoxia induces a stronger transcriptional response in NMR hearts compared to mice, indicating an enhanced capacity to rapidly reprogram gene expression in response to stress (Fig. 23A and B). Functional enrichment analysis further revealed that hypoxia-responsive genes in NMR hearts are mainly associated with transcriptional regulation, as well as processes related to cell development, proliferation, and differentiation (Fig. 23C). Neonatal CM-associated genes are significantly enriched among hypoxia-upregulated genes, and a subset of up-regulated genes under hypoxia in NMR hearts overlaps with those highly expressed in P1 mouse hearts (Fig. 23E). These observations suggest that responses in NMR hearts promote a shift toward a more neonatal-like transcriptional state.

Previous studies have suggested that hypoxia could promote cardiac regeneration[74]. Consistent with this, hypoxia induces the activation of transcription regulators in NMR heart associated with cardiac development and regeneration, including *Pitx2*, *Hey2*, *Sfrp1*, *Wt1*, *Tgif2*, *Myc* and *Id2* (Fig. 23C and E). Importantly, several transcription factors, including *Wt1*, *Tgif2* and *Myc*, are closely associated with EMT-related signaling, developmental plasticity, and cellular reprogramming[219–221], suggesting that hypoxia in NMR hearts activates a broader transcriptional network linked to regenerative adaptation.

However, the hypoxic response in NMR hearts extends beyond a simple reactivation of neonatal-like gene expression programs and instead involves species-specific regulatory features. In particular, components of the AP-1 transcription factor complex, especially members of the FOS family, are markedly upregulated under hypoxia in NMR hearts but are not in neonatal hearts (Fig. 23J). Interestingly, motif enrichment analysis revealed significant enrichment of AP-1 and Tgif2-binding motifs in NMR-specific more-active accessible enhancers under normoxic conditions (Table S3). This pre-existing regulatory landscape may provide a molecular basis for the rapid transcriptional response of NMR hearts under stress conditions.

In addition, hypoxia induces broad activation of EMT-associated programs in NMR hearts (Fig. 25E). EMT-related pathways are closely linked to cellular plasticity, cytoskeletal remodeling, extracellular matrix reorganization, and regenerative responses[150, 222, 223]. Notably, AP-1 signaling has been shown to cooperate with EMT-associated transcriptional programs[87], while ERBB2 activation has also been shown to promote EMT-like processes during cardiac regeneration. These observations suggest that ERBB2 signaling in NMR hearts may not be activated exclusively in response to injury but may also contribute to hypoxia-induced stress responses. Therefore, AP-1 activation and ERBB2 signaling may act in concert under hypoxic conditions to promote EMT-associated transcriptional programs and cellular remodeling. Taken together, these findings suggest that stress-responsive signaling networks in NMR hearts establish a more plastic and regeneration-permissive cellular state under stress conditions.

5.5 Bivalent chromatin architecture enables rapid gene activation under stress
Bivalent promoters are typically marked by the activating histone modification H3K4me3 while simultaneously carrying the repressive histone mark H3K27me3. Importantly, previous studies have demonstrated that these two histone modifications can coexist in the same DNA regions, indicating that bivalent chromatin is not merely a consequence of cellular heterogeneity but rather represents a distinct regulatory state that determines lineage-specific gene expression during development[122, 224]. This chromatin state was initially identified in embryonic stem cells, where developmental genes remain transcriptionally silent yet poised for rapid activation[122].

Although bivalent promoters are progressively resolved during mammalian differentiation and maturation, we found that a subset of genes in both adult NMR and mouse hearts retain a bivalent chromatin state (Fig. 24D). Interestingly, under hypoxic conditions, NMR hearts exhibit stress-induced reactivation of 104 poised genes, whereas similar transcriptional activation is largely absent in mouse hearts (Fig. 25A). Rather than representing random genes, these hypoxia-responsive bivalent genes are strongly associated with biological processes involved in cardiac and vascular development, extracellular matrix remodeling, cell migration, and stress-responsive signaling pathways (Fig. 25B). While persistent activation of these pathways in adult mammalian hearts frequently contributes to pathological fibrosis, regenerative systems

often utilize transient extracellular matrix remodeling to support structural reorganization and tissue recovery[225]. Therefore, the transcriptional activation observed in NMR hearts may reflect a controlled adaptive response rather than irreversible pathological remodeling. These findings also suggest that the persistence of bivalent chromatin domains in adult NMR hearts may provide an epigenetic basis for the enhanced transcriptional plasticity and stress responsiveness.

However, we do not currently have histone mark ChIP-seq data from hypoxic NMR or mouse hearts. Therefore, although these genes exhibit bivalent chromatin states under normoxic conditions and are transcriptionally activated under hypoxia in NMR hearts, we cannot definitively conclude that their stress-induced expression is directly mediated through reactivation of bivalent promoters. Nevertheless, the selective induction of these poised genes in NMR hearts, but not in mouse hearts, strongly supports the idea that the retained bivalent chromatin architecture in NMRs contributes to enhanced transcriptional responsiveness under environmental stress conditions.

Together, these findings support a model in which adult NMR hearts retain a partially permissive chromatin landscape that may enable stress-induced activation of developmental and regenerative pathways. This regulatory flexibility represents an important mechanism underlying the exceptional stress tolerance and regenerative potential of the NMR heart.

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Appendix

Fig. S1 Schematic overview of the identification of differentially regulated genes.

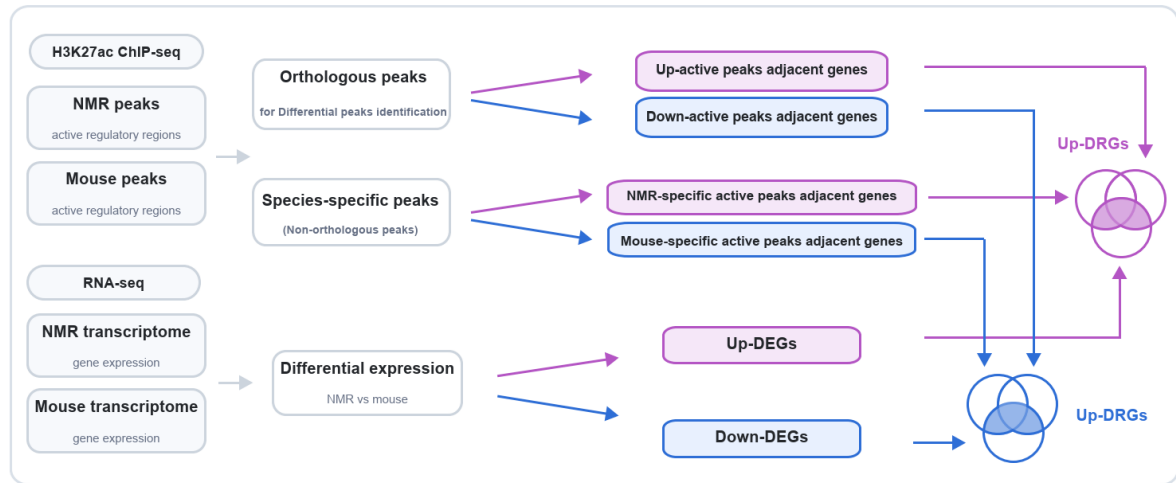


Table S1. Gene list of 175 upregulated neonatal-like genes.

Myh7	Folr1	Csrp1	Sntg2	Cenps	Pycr1	Iqgap3
Myh8	Frmpd1	Cthrc1	Soat1	Cenpu	Pygl	Iqschfp
Myoz1	Frrs1	Ctxn1	Spats2	Cenpw	Pyroxd2	Jazf1
Ncam1	Ftl1-ps2	Cyp46a1	Spin2c	Cfap20dc	Rab33a	Jpt2
Nckap5l	Fzd3	Dbn1	Sqle	Chaf1a	Rad51	Kdelr3
Nek2	Gale	Dclk2	Stbd1	Chek1	Rad51ap1	Kif11
Nek6	Gins2	Dctd	Stxbp2	Cirbp	Ranbp17	Kif4
Ngfr	Gjd3	Dhcr24	Tead2	Cited1	Rbm3	Klhdc8b
Nnat	Gpc3	Dpysl3	Tgfb1i1	Ckap2	Rec114	Krt8
Npas2	Gprc5a	Dscc1	Tgif2	Cks1b	Renbp	Lamc2
Nrep	Gpsm2	Dsn1	Tmem254	Cks2	Rfc5	Ldlr
Pafah1b3	Gpx7	Dynll1	Tnni1	Cldn11	Ripk3	Lig1
Pcbd1	Greb1l	Efcab11	Tpbpg	Cma1	Ripor2	Mad2l1
Pcdhga8	Hand1	Eif4ebp3	Tpx2	Col11a1	Rnf26rt	Map7d3
Pdk3	Haus4	Eme1	Traf5	Col12a1	Sdk1	Mcm3
Pheta2	Hddc2	Eno1b	Tuft1	Col13a1	Septin6	Mcm6
Phldb2	Hebp2	Ephx4	Tyms	Col9a2	Sinhcaf	Mgst2
Pld6	Hif3a	Erbp2	Tyro3	Cotl1	Slc16a3	Msln
Pole2	Hmgb2	Fam167b	Ubash3b	Cpz	Slc22a17	Mxd3
Ppfia4	Hmgn3	Fancd2	Wdr62	Crmp1	Slc6a13	Myc
Ppp1r1a	Idi1	Fanci	Zfp105	Cdh11	Cd276	Cachd1
Ppp1r3g	Igf2bp2	Firm	Zfp184	Cenph	Cdc6	Ccdc112
Psrc1	Igfbp2	Fkbp1b	Zfp711	Cenpn	Cdca7l	Cd109
Bex2	Arsg	Arhgap28	Ager	Adamts14	A2m	1500011B03Rik
Bid	Asf1b	Armxc6	Alcam	Adnp	Acsl3	9930012K11Rik

Table S2. Gene list of 291 downregulated neonatal-like genes.

Hk2	Ifit1	Rasl10a	Prex2	Stum	Klhdc1	Vit
Gstt1	Gck	Atp8a1	Ctla4	St6galnac2-1	Arhgap20	Psmb8
Il16	Cntfr	P2ry2	Slc9a2	Togaram2	Dand5	Fas
Arrdc2	Cngb3	Txlnb	Ptpn	Fsd1l	Ech1	Doc2g
Nqo1	Krt222	Slc25a34	Coq8a	Slc37a2	Kcnk3	Papss2
Mtfp1	Sgca	Csdc2	Efcab2	Herpud1	Acox1	C1qtnf9
Mlxip1	Dcn	Slc36a2	Plxdc2	Kcnip2	Mettl7a1	Cbr2
Fblim1	Ubxn10	Chad	Agpat2	Cd200	Pcx	Cyp2e1
Arhgef19	Trim72	H2-Aa	Entpd2	Tecr	Rnf144b	Clec3b
Snai3	Podn	Maob	Xirp2	Cyfp2	Tmem143	Arhgap26
Clec3a	Art5	Psme4	Chac1	Dnajc28	Penk	Cped1
Apip	Ak4	Lrg1	Ivd	Ccdc159	Ephx2	Mid1ip1
Trim54	Fmo5	Serpine1	Car3	Chrm2	Osbp2	Tango2
Pkd2l2	Tmod4	Ppm1k	P2ry1	Nim1k	Trpv4	Magix
Csf1	Fam174b	Scn7a	Tmem144	Sbk2	Cyt1l	Fam107a
Pcolce2	Plxdc1	Cebpa	Ptgfr	Gvin1	Acyp2	Dpysl4
Lpl	Tmem245	Thrsp	F3	Tac1	Drd2	Myo5b
Hsd11b1	Kcna7	Coq10a	Aldob	Misp3	Srrm4	Sbk3
Vtn	Cp	Grm1	Cavin4	Strit1	Rnf150	Susd5
Rhbdl3	Acsl6	Fbxl16	Alpl	Rgs7	St6galnac2	Cd74
Dpep1	Pltp	Fmo2	Prxl2b	Sds	Cpeb3	Kcnv2
Plin5	Fitm2	Gpc1	Yipf7	Klf15	Dbp	Cilp2
Tcp11l2	Tnfaip2	Fmo1	Cds1	Cyp26b1	Oas2	Pdp2
Best3	Dglucy	Nceh1	Crybb1	Kcnb1	Kcnd2	Itgb6
Cpm	Shld1	Abcb1a	Ppbp	Cox7a1	0610040J01Rik	Tmtc1
Ccng1	Gpcpd1	Acad11	Pxmp2	Il18bp	Htra4	Scn4b
Nampt	Slc28a2	Prep	Clcn1	Slc17a7	Whrn	Gal3st3
Cxcl14	Ntsr2	Cilp	Eif4e3	Art1	Hrc	Trarg1
Agtpbp1	Nr1h3	Mreg	Plbd1	Smco4	Ttc32	Hamp
Galnt15	Creg1	Hjv	Timp4	Ptprb	Atp1a2	Rom1
Ogdhl	Ccl2	Itgbl1	Rtn2	Omd	Glul	Egln1
Clu	Cfh	Igfals	Ucp3	Pgf	Klhl34	Mmp3
Tef	Cryba4	Mterf2	Dgat2	Lrtm1	Lnx1	Rora
Lynx1	Cd163	Tmem82	Cox6a2	Aldh1a1	Cd86	Lrrc2
Lypd2	Pfkfb1	Lingo3	Vwa3a	Klhl33	Plaat1	Pygm
Adipoq	Cxcl9	Abra	Asb5	Klf12	Sybu	Fibin
Sncg	Prrg1	Lrrc3b	Gpt2	Tafa5	Nr1d2	Gpr27
Ivns1abp	Zbtb20	Zfp106	Il15	Smoc2	Rapgef4	Pla2g5
Slc22a3	Hadhb	Car7	Ces1d	Tbc1d10c	Phyhd1	Cyp4b1
Gpam	Intu	Fndc5	Ogdh	Kiz	Armc2	Wfdc17
Prr33	Lyz2	Aqp8	Trabd2b	Hcn2	3425401B19Rik	Smco1
Amd1	Mylk4	Gsta1	Mypn			

Table S3. TOP10 motif enrichment result of Up- and Down- active accessible peak sets.

Motif enriched in NMR more-active accessible promoters			
Rank	Motif Name	Consensus	P-value
1	Mef2d(MADS)/Retina-Mef2d-ChIP-Seq(GSE61391)/Homer	GCTATTTT TAGC	1.00E-38
2	Mef2c(MADS)/GM12878-Mef2c-ChIP-Seq(GSE32465)/Homer	DCYAAAAA TAGM	1.00E-32
3	bHLHE40(bHLH)/HepG2-BHLHE40-ChIP-Seq(GSE31477)/Homer	KCACGTGMCN	1.00E-23
4	CTCF(Zf)/CD4+-CTCF-ChIP-Seq(Barski_et_al.)/Homer	AYAGTGCCMYCTRGTGGCCA	1.00E-23
5	BORIS(Zf)/K562-CTCF-ChIP-Seq(GSE32465)/Homer	CNNBRGCGCCCCCTGSTGGC	1.00E-22
6	Mef2a(MADS)/HL1-Mef2a.biotin-ChIP-Seq(GSE21529)/Homer	CYAAAAA TAG	1.00E-20
7	Mef2b(MADS)/HEK293-Mef2b.V5-ChIP-Seq(GSE67450)/Homer	GCTATTTT TGGM	1.00E-19
8	bHLHE41(bHLH)/proB-Bhlhe41-ChIP-Seq(GSE93764)/Homer	KCACGTGMCN	1.00E-17
9	USF1(bHLH)/GM12878-USF1-ChIP-Seq(GSE32465)/Homer	SGTCACGTGR	1.00E-16
10	RFX(HTH)/K562-RFX3-ChIP-Seq(SRA012198)/Homer	CGGTTGCCATGGCAAC	1.00E-16
Motif enriched in mouse more-active accessible promoters			
Rank	Motif Name	Consensus	P-value
1	Mef2d(MADS)/Retina-Mef2d-ChIP-Seq(GSE61391)/Homer	GCTATTTT TAGC	1.00E-82
2	Mef2c(MADS)/GM12878-Mef2c-ChIP-Seq(GSE32465)/Homer	DCYAAAAA TAGM	1.00E-61
3	Mef2b(MADS)/HEK293-Mef2b.V5-ChIP-Seq(GSE67450)/Homer	GCTATTTT TGGM	1.00E-51
4	Mef2a(MADS)/HL1-Mef2a.biotin-ChIP-Seq(GSE21529)/Homer	CYAAAAA TAG	1.00E-49
5	BORIS(Zf)/K562-CTCF-ChIP-Seq(GSE32465)/Homer	CNNBRGCGCCCCCTGSTGGC	1.00E-31
6	Tlx?(NR)/NPC-H3K4me1-ChIP-Seq(GSE16256)/Homer	CTGGCAGSCTGCCA	1.00E-24
7	CTCF(Zf)/CD4+-CTCF-ChIP-Seq(Barski_et_al.)/Homer	AYAGTGCCMYCTRGTGGCCA	1.00E-22
8	NF1(CTF)/LNCAP-NF1-ChIP-Seq(Unpublished)/Homer	CYTGGCABNSTGCCAR	1.00E-16
9	GRE(NR),IR3/RAW264.7-GRE-ChIP-Seq(Unpublished)/Homer	VAGRACAKWCTGTYC	1.00E-13
10	GFX(?)/Promoter/Homer	ATTCTCGCGAGA	1.00E-11
Motif enriched in NMR more-active accessible enhancers			
Rank	Motif Name	Consensus	P-value
1	Mef2d(MADS)/Retina-Mef2d-ChIP-Seq(GSE61391)/Homer	GCTATTTT TAGC	1.00E-194
2	Mef2c(MADS)/GM12878-Mef2c-ChIP-Seq(GSE32465)/Homer	DCYAAAAA TAGM	1.00E-191
3	Mef2b(MADS)/HEK293-Mef2b.V5-ChIP-Seq(GSE67450)/Homer	GCTATTTT TGGM	1.00E-184
4	Mef2a(MADS)/HL1-Mef2a.biotin-ChIP-Seq(GSE21529)/Homer	CYAAAAA TAG	1.00E-168
5	NF1(CTF)/LNCAP-NF1-ChIP-Seq(Unpublished)/Homer	CYTGGCABNSTGCCAR	1.00E-84
6	NF1-halfsite(CTF)/LNCaP-NF1-ChIP-Seq(Unpublished)/Homer	YTGCCAAG	1.00E-76
7	Tlx?(NR)/NPC-H3K4me1-ChIP-Seq(GSE16256)/Homer	CTGGCAGSCTGCCA	1.00E-47
8	Jun-AP1(bZIP)/K562-cJun-ChIP-Seq(GSE31477)/Homer	GATGASTCATCN	1.00E-44
9	Fosl2(bZIP)/3T3L1-Fosl2-ChIP-Seq(GSE56872)/Homer	NATGASTCABNN	1.00E-40
10	Tgif2(Homeobox)/mES-Tgif2-ChIP-Seq(GSE55404)/Homer	TGTCANYT	1.00E-39
Motif enriched in mouse more-active accessible enhancers			
Rank	Motif Name	Consensus	P-value
1	Mef2c(MADS)/GM12878-Mef2c-ChIP-Seq(GSE32465)/Homer	DCYAAAAA TAGM	1.00E-284
2	Mef2d(MADS)/Retina-Mef2d-ChIP-Seq(GSE61391)/Homer	GCTATTTT TAGC	1.00E-267
3	Mef2b(MADS)/HEK293-Mef2b.V5-ChIP-Seq(GSE67450)/Homer	GCTATTTT TGGM	1.00E-258
4	Mef2a(MADS)/HL1-Mef2a.biotin-ChIP-Seq(GSE21529)/Homer	CYAAAAA TAG	1.00E-205

5	NF1(CTF)/LNCAP-NF1-ChIP-Seq(Unpublished)/Homer	CYTGGCABNSTGCCAR	1.00E-120
6	NF1-halbsite(CTF)/LNCaP-NF1-ChIP-Seq(Unpublished)/Homer	YTGCCAAG	1.00E-83
7	Tlx?(NR)/NPC-H3K4me1-ChIP-Seq(GSE16256)/Homer	CTGGCAGSCTGCCA	1.00E-62
8	GRE(NR),IR3/A549-GR-ChIP-Seq(GSE32465)/Homer	NRGVACABNVTGTICY	1.00E-61
9	ERRg(NR)/Kidney-ESRRG-ChIP-Seq(GSE104905)/Homer	GTGACCTTGRVN	1.00E-58
10	Esrrb(NR)/mES-Esrrb-ChIP-Seq(GSE11431)/Homer	KTGACCTTGA	1.00E-57

Table S4 29 NMR fast-evolving elements on *Esrrg* gene body (location on hg38).

chr	start	end
chr1	216,596,413	216,596,433
chr1	216,606,149	216,606,245
chr1	216,606,632	216,606,774
chr1	216,652,042	216,652,163
chr1	216,656,392	216,656,415
chr1	216,656,563	216,656,688
chr1	216,677,699	216,677,760
chr1	216,677,768	216,677,794
chr1	216,679,323	216,679,379
chr1	216,679,469	216,679,485
chr1	216,680,655	216,680,682
chr1	216,682,998	216,683,081
chr1	216,683,082	216,683,224
chr1	216,688,351	216,688,494
chr1	216,705,485	216,705,501
chr1	216,705,741	216,705,802
chr1	216,705,966	216,705,995
chr1	216,722,611	216,722,681
chr1	216,722,724	216,722,789
chr1	216,722,854	216,723,017
chr1	216,723,218	216,723,360
chr1	216,723,363	216,723,538
chr1	216,723,771	216,723,799
chr1	216,740,121	216,740,306
chr1	216,780,289	216,780,311
chr1	216,830,371	216,830,394
chr1	216,916,112	216,916,158
chr1	217,089,852	217,090,004
chr1	217,138,515	217,138,615

Table S5 Gene list of 147 Down-DRGs also as ERR-activated targets.

Dpysl4	Gimap5	Actr3B	Fndc5	Kremen1	Etfb	Tnfaip8
Icam2	Fxn	Tnfaip2	Kank3	Khdrbs3	Cep68	Pygm
Lipt1	Ntn1	Itgb6	Mccc1	Gpsm1	Acadm	Art4
Reep5	Kcnb1	Tmem143	Adk	Podxl	Clec3b	Kifc3
Hmgcs2	P2ry1	Kcnn2	Nampt	Slc47a1	Flvcr2	Ank1
Smco4	Fitm2	Tmc7	Hcn2	Vwa8	Ackr4	Kcnk3
Cyp4b1	Cpt2	Selenbp1	Chrm2	Polr2m	Uaca	F3
Hadhb	Hadh	Agtpbp1	Cmtm8	Smco1	Kif16b	Whrn
Grm1	Cyp26b1	Btbd3	Trabd2b	Pdk2	Tmem182	Slc25a39
Slc28a2	Ivd	Ogdh	Akap7	Bmper	Chac2	Msra
Sh3d21	Coq8a	Dock8	Cadm4	Fam107a	Pccb	Clasp1
Apln	Aqp7	Nt5dc3	Abhd18	Ankrd50	Tmem106b	Gramd1b
Pptc7	Ggact	Fhl2	Dand5	Rap1gap	Cox6A1	Xirp2
Ogdhl	Dcakd	Scarb1	Papln	Gpt2	Klf15	Gpcpd1
Il15	Macrod1	Kcna5	Spsb4	Cpeb3	Bckdhb	Raph1
Clcn1	Slc25a13	Lrtm1	Homer2	Sgcg	Ppp2r3A	Sbk1
Mxi1	Lpl	ErbB3	Prdx5	Hmgb3	Tcp11l2	Tmem171
St3gal3	Pik3r6	Acacb	Decr1	Ddc	Pparg	Sord
Plxdc1	Asrgl1	Lclat1	Ccnjl	Epb41	Rnf150	Klhl3
Tmem135	Slc41a3	Rhbdl3	Map7	Sucla2	Nrp2	Rassf3
Ndufa13	Trak2	Il15ra	Dym	Plin3	Efcab2	Esrb

Table S6 Gene list of 67 Up-DRGs also as ERR- suppressed targets.

Hpn	Ncam1	Frzb	Pbdc1	Rab15	Dbn1	Cntn5
Gas7	Gp1bb	Nipsnap3b	Adamtsl1	Pygl	Sh3bp4	Pi16
Maoa	Mcm3	Ttc39a	Pemt	Cyp39a1	Ccdc112	Tsku
Ddn	Hr	Mthfd1l	Col12a1	Bhlhe41	Pcbd1	Efhc1
Tnfrsf11b	Fgr	Wfdc3	Sh3d19	Vwf	Slc4a4	Enpp1
Grk5	Gpx1	Adcy5	Tpm2	Stbd1	Atf3	Dok5
Tspan17	Dtwd1	Dera	Armxc6	Col13a1	C7	Lamc2
Ppp1r3c	Anxa1	Cers6	Sh3bp2	Polm	Frrs1	Synm
Syne3	Ubash3b	Nrtn	Loxl4	Dhcr24	Slc16a3	Hps1
Krt8	Paqr6	Unc13b	Tnni1			