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Adenylate kinase AK2 isoform integral in embryo and adult heart homeostasis

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Abstract

Adenylate kinase 2 (AK2) catalyzes trans-compartmental nucleotide exchange, but the functional implications of this mitochondrial intermembrane isoform is only partially understood. Here, transgenic AK2−/− null homozygosity was lethal early in embryo, indicating a mandatory role for intact AK2 *in utero* development. In the adult, conditional organ-specific ablation of AK2 precipitated abrupt heart failure with Krebs cycle and glycolytic metabolite buildup, suggesting a vital contribution to energy demanding cardiac performance. Depressed pump function recovered to pre-deletion levels overtime, suggestive of an adaptive response. Compensatory upregulation of phosphotransferase AK1, AK3, AK4 isozymes, creatine kinase isoforms, and hexokinase, along with remodeling of cell cycle/growth genes and mitochondrial ultrastructure supported organ rescue. Taken together, the requirement of AK2 in early embryonic stages, and the immediate collapse of heart performance in the AK2-deficient postnatal state underscore a primordial function of the AK2 isoform. Unsalvageable in embryo, loss of AK2 in the adult heart was recoverable, underscoring an AK2-integrated bioenergetics system with innate plasticity to maintain homeostasis on demand.

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Conflict of Interest Statement.

The authors listed below declare no conflict of interest pertinent to this manuscript.

Declaration of Competing Interest

The authors declare no competing financial interests.

Keywords

Adaptation; Adenylate kinase; Homeostasis; Knockout; Metabolism; Phosphotransfer

1. Introduction

The adenylate kinase (AK) system, consisting of multiple isoforms (AK1-AK9), is implicated in cell energetics and metabolic signaling [1–6]. AK enzymes catalyze the ATP +AMP ↔ ADP+ADP reaction in response to intracellular ATP and ADP fluctuations [1]. Answering to variations in workload, oxygen tension or energy metabolism, the AK-centric network is thought to generate and convey adenine nucleotide signals to metabolic sensors [5,7–9]. AK isoforms have a unique proficiency of using both γ- and β-phosphoryls in the ATP molecule, thereby doubling the ATP energetic potential and contrasting traditional γ-only utilizing enzymes [1,2,10]. The three major isoforms, namely AK1, AK2, and AK3, are localized in the cytosol, mitochondrial intermembrane space, and matrix, respectively, facilitating transcellular nucleotide exchange [3,5,9]. To date, the emphasis has been placed on cytosolic AK1 (known as myokinase), and its mitochondrial matrix AK3 alternative [11–14], yet less is known regarding the function of the AK2 isoform.

AK2 is critically positioned in the mitochondrial intermembrane/intra-cristae space, where it is postulated to serve at the crossroad of the AK phosphotransfer network [1,2,5]. AK2 mutations have been associated with rare congenital human diseases linked to early mortality, immune deficiency and hearing loss [15–18]. Molecular studies indicate that AK2 participates in cell fate decisions and nuclear energetics [1,3,9,19]. In fact, in energy demanding organs, such as the heart characterized by high energy turnover and an elaborate mitochondrial circuit, AK2 appears to accounts for ~40% of total AK activity [20,21]. To avoid confounding influences of related enzymes, in particular in complex circuits of energy demanding tissue, establishing isoform- and organ-specific models would streamline the decoding of AK2-dependent biology.

To this end, here, global germline AK2 knockout and heart-specific conditional AK2 transgenic mice were generated. Complete deletion of the AK2 gene was embryonically lethal, indicating a prerequisite for intact AK2 in support of developmental programming. Cardiac-specific AK2 disruption in the adult, fully developed heart temporarily stunned contractile performance, with recovery enabled by an orchestrated adaptive metabolic rebound underscoring AK2 integration within a resilient bioenergetic ecosystem.

2. Materials and Methods

All animal experiments were carried out in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and with approval from the Institutional Animal Care and Use Committee. Male and female animals were used in the study.

2.1. Generation of global AK2 knockout mice

Adenylate kinase AK2 germline gene knockout mice (AK2−/−) were generated using the embryonic stem cell clone OST495245, which harbors the TG0053(Ak2) gene trapping

vector VICTR CREATR 628 within the first intron of AK2 (GenBank accession number: NM_016895; Texas A&M Institute for Genomic Medicine). Mice were maintained on a mixed 129/SvEv-C57BL/6N background.

2.2. Generation of conditional heart-specific transgenic AK2

Conditional heart-specific transgenic AK2 was produced by deleting exon 3 and 4 using Cre-Lox recombination and CRISPR/Cas9 technology (Cyagen). The targeting vector, containing LoxP, selectable marker Neomycin with Frt, exon 3 and 4, and LoxP, was transfected into embryonic stem cells. Identified positive stem cells were injected into blastocysts, and resulting mice mated with Flp deleter to generate conditional heart-specific transgenic AK2 offspring. Generated mice were utilized to produce heart-specific AK2 gene deletion by crossing with cardiac-specific tamoxifen-inducible Cre-mediated recombinase mice, B6.FVB(129)-A1cfTg(Myh6-cre/Esr1*)1Jmk/J. Progenies were crossed to obtain homozygotes of conditional tamoxifen-inducible heart-specific transgenic AK2 knockout, which were intraperitoneally injected for 5 days with 1 mg/day of tamoxifen (Sigma T5648; suspended in 0.5 mL of ethanol and 9.5 mL of corn oil) at 6–8 weeks of age for programmed heart-specific AK2 deletion in the adult. In pre-screening of cre+/+ and flox−/− mice, the tamoxifen regimen did not affect cardiac performance (data not shown). Separately, age- and sex-matched AK2−/− mice were injected with an ethanol/corn oil mixture to serve as sham control.

2.3. Echocardiography

In adult wild-type and transgenic mice, cardiac function and structure were evaluated using transthoracic echocardiography with a 30-MHz probe (Vevo3100, FUJIFILM VisualSonics) under isoflurane anesthesia (1–2%). Left ventricular chamber size and contractility were prospectively followed [22, 23].

2.4. Metabolites

Metabolite levels in heart perchloric acid extracts were measured using HPLC and GC-MS (Agilent 5980B) as previously established [9].

2.5. Genotyping and gene expression analysis

Mice were genotyped for AK2 gene deletion utilizing the following primers: Primer TG0053–5’ CCTCGGAGTTGAGACGCCGTATT, Primer LTR-rev ATAAACCCTCTTGCAGTTGCATC and Primer TG0053–3’ CAATCCTTGAGGCCTCTGTGAAACC. Heart RNA was isolated using the RNeasy kit (QIAGEN). Equal quantities of RNA from each sample were reverse transcribed into cDNA using the SuperScript III Reverse Transcriptase and oligo dT(18) primers. Quantitative real-time PCR (qPCR) was performed using the TaqMan™ Universal PCR Master Mix (ThermoFisher Scientific). Standard primer sequences were used for genes of interest. Expression of target genes was normalized to gapdh expression.

2.6. Western blots

Western blots were done using specific anti-AK antibodies (Abcam).

2.7. Ultrastructure

Cell ultrastructure was assessed by transmission electron microscopy [24].

2.8. Statistics

Data are expressed as mean±SEM, and analyzed using Statistica software (StatSoft). The Student's t-test for unpaired samples was used, and a difference at $P<0.05$ considered significant.

3. Results

3.1. Germline AK2 gene deletion causes embryonic lethality

AK2 ablation was engineered using an embryonic stem cell gene trapping method disrupting the first intron of *AK2* (Fig. 1A). Breeding AK2+/− heterozygote mice produced an unexpected 36% wild-type (WT) and 64% AK2+/− offspring ratio, with no surviving AK2-null (AK2−/−) homozygotes (Fig. 1B). Lethality of AK2−/− embryos was timed at ~7 days, and confirmed by genotyping (Fig. 1C & 1D). Notably, on autopsy, AK2−/− embryos (E7.5) displayed a severely disrupted and disorganized mitochondria and cristae ultrastructure, contrasting heterozygous AK2+/− or WT counterparts (Fig. 1E). Thus, loss of AK2, residing in mitochondrial intermembrane/intra-cristae, translates into disruption of early embryo development.

3.2. Adult cardiac-specific AK2 deletion provokes organ decompensation with recovery

To overcome *in utero* lethality, conditional cardiac-specific AK2−/− null mice were engineered (Fig. 2A). In transgenic adults (6 to 8-week old), programmed cardiac AK2 knockout was induced by tamoxifen challenge, with loss of expression demonstrated on immunoblots (Fig. 2B). In contrast to no change in induced WT and non-induced AK2−/− controls, within 1-week post-induction, AK2−/− mice displayed pathologic ventricular dilation, measured as an increase in end-diastolic and end-systolic volumes, and a compromise in force generation, measured by a decrease in left ventricular ejection fraction (Fig. 2C–2G). Cardiac chamber enlargement and reduced pump function were however transitory, and recovered to pre-induction levels within 2 months (Fig. 2C–2G). Consistent with the rescue of mechanical function, myocardial ultrastructure, including mitochondrial cristae, were on follow-up examination indistinguishable from WT counterparts (Fig. 2H). Thus, in the developed adult heart, abrupt AK2 loss produced acute organ failure, implicating intact AK2 in cardiac homeostasis. Recovery of malfunction overtime, despite the AK2 depleted state, suggests an underlying bioenergetics adaptive resilience.

3.3. Adaptive Response of AK2 ablated heart

To gain insight into the molecular malleability underlying the long-term heart rescue, gene expression and metabolite profiles of phosphotransfer, mitochondrial, and cell cycle/growth pathways were probed at the decompensated (1 week), and then at the recovery (at 12 week) phases, post-AK2 ablation. Induction of the AK2−/− state provoked an acute (within 1 week) depression of the AK isoform gene family, with notable rebound/up-regulation of *ak1*, *ak3*, and *ak4* isoforms at follow-up (within 12 weeks) reaching levels of WT counterparts

(Fig. 3A). Similarly, genes for cytosolic and mitochondrial creatine kinase (CK) isoforms (*ckmt2*, *ckm* and *ckb*), nucleoside diphosphate kinase (NDPK), and hexokinase isoforms (*hk1* and *hk2*), responded with transient down-regulation on AK2 ablation followed with recovery on follow-up (Fig. 3B & 3C), indicating a compensatory collective expression pattern across phosphotransfer enzyme dynamics. Moreover, expression of genes (*opa1* and *dnm1*) involved in regulating mitochondrial morphogenesis also declined temporarily followed by timely recovery that matched WT (Fig. 3D). In response to genetic stress induced by AK2 deletion, an early overexpression of cell cycle and growth genes (*p53*, *ccne1* and *dusp26*) was detected, which over-time returned to pre-AK2 ablation levels (Fig. 3E) suggestive of myocardial normalization. A rise of Krebs cycle metabolites, fumaric and malic acids, along with the glycolytic metabolite glucose-6-phosphate, further implicated following AK2 deletion coordinated, multivalent energy metabolism adjustments. Thus, the abrupt heart-specific loss of AK2 triggered a synchronized genomic and metabolic remodeling, underscoring an on-demand plasticity and homeostatic adaptability that innately protects the AK2 constitutively deficient myocardium.

4. Discussion

The present study reveals a previously unrecognized vital role for the adenylate kinase isoform 2 (AK2) in prenatal and postnatal homeostasis. Recognized physicochemically as a phosphotransfer enzyme that catalyzes trans-compartmental nucleotide exchange, the functionality of AK2 in the embryo and adult had remained only partially addressed [15–18]. To gain further insight, both global constitutive and organ-specific conditional gene knockout models were here developed to probe for AK2 impact during development and in adulthood. Lack of AK2 precipitated a pronounced developmental liability causing early embryo lethality. In the adult, programmed cardiac-specific ablation of AK2 produced instant, yet transient, heart malfunction triggering a preserving molecular response. The mandatory requirement for AK2 in early embryogenesis and the massive remodeling to safeguard an energy demanding organ in the adult underscore an obligatory, primordial phosphotransferase that evolves during life within an energy metabolism system harboring build-in plasticity apt to ensure long-term resilience.

Among multiple adenylate kinase isoforms, AK1 (or myokinase) has been the most recognized contributor in the maintenance of cellular phosphotransfer energetics. In contrast, the function of AK2, positioned at the juncture of the mitochondrial intermembrane/intra-cristae space, had remained elusive. Herein, transgenic AK2−/− null homozygosity was found to be lethal *in utero*, reflecting a severe congenital developmental vulnerability associated with lack of the AK2 isoform. In this regard, the present study extends and generalizes, using a mammalian species, the impact of AK2 deficiency in limiting survival, disturbing morphogenesis, and compromising development previously observed in larvae of *Drosophila melanogaster,* or in yeast where AK2 deletion shuts down mitochondrial energetics [4, 25]. This obligatory requisite points to the uniqueness of the AK2 catalyzed function in the early embryo, a property apparently not interchangeable with AK1 or other adenylate kinase isoforms [26]. In fact, during early development, the strategically mapped AK2 worked as a bottleneck for phosphotransfer conduits, and was unable to be replaced by a different and compensatory enzyme and/or route. The ensuing embryo mortality observed

here was timed to day 7, which coincides in schedule with the development of the very first organ, namely the heart, thus channeling energy metabolism to match supply and demand. Elucidation of the isoform that underpins phosphotransfer catalyzed circuits in nascent organ development positions AK2 as a gatekeeper in serving a major primal role.

Unlike a mandatory involvement in early development ensuring embryo survivorship, cardiac-specific AK2 deletion in the already developed adult heart was not here associated with increased lethality. Rather, organ-specific ablation in the matured animal precipitated a transient heart derangement with substantial gene expression remodeling associated with a timely rescue of cardiac pump function. Notably, in response to abrupt AK2 deletion, multiple phosphotransfer enzymes, including AK and CK isoforms, NDPK, and hexokinase, were all only temporarily downregulated. To compensate for the lack of AK2, however, these enzyme systems became collectively upregulated ensuring restorative transition, and thus underscoring a complementary and inter-connectivity among phosphotransfer network members in the adult heart [27]. Furthermore, the increased expression of cell cycle and growth genes within a week of AK2 ablation indicated an enhancement of cell proliferation supporting early adaptation, and regressed ultimately to wild-type levels signaling reacquired homeostasis. This is in line with enhanced cell proliferation observed in response to targeted disruption of AK2 expression precipitated by short interfering RNAs [28]. Moreover, the fail-safe operation of intracellular bioenergetics, supporting a broad range of cellular functions, is fundamental for tightly coupled adenine nucleotide producing and consuming biological processes [29]. Accordingly, deleting other high-energy phosphoryl transferases, organized within a spatially arranged enzymatic network, also provokes a genetic and metabolic remodeling leading to utilization of alternative phosphoryl transfer routes [30]. Taken together, the adult heart harbors thus a remarkable aptitude to rebound from single gene phosphotransfer enzyme deletion, relying on a robust adaptive remodeling.

In summary, here, the engineered isoform/organ-specific transgenic models served to unmask the AK2-dependent prenatal and postnatal biology. Remarkably, AK2 was revealed vital and unreplaceable in support of early embryogenesis. While intact AK2 was mandatory for *in utero* development, in the adult heart, the observed plasticity of the phosphotransfer network was permissive in achieving sustenance of organ performance within an AK2-depleted milieu. Thus, AK2 evolves from compulsory *in utero* to able to trigger a compensatory response in adult life, unveiling an increasingly malleable bioenergetic system working in unison to ensure organ wellbeing.

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Highlights

- The role of phosphotransferase adenylate kinase 2 (AK2) is unknown
- Here, AK2 deletion was found lethal *in utero*, implicating requirement in embryogenesis
- Conditional AK2 ablation in developed heart produced instant organ malfunction
- AK2-dependent cardiac pump failure salvage required genetic and metabolic remodeling
- Adult energy metabolism harbors build-in plasticity ensuring resilience

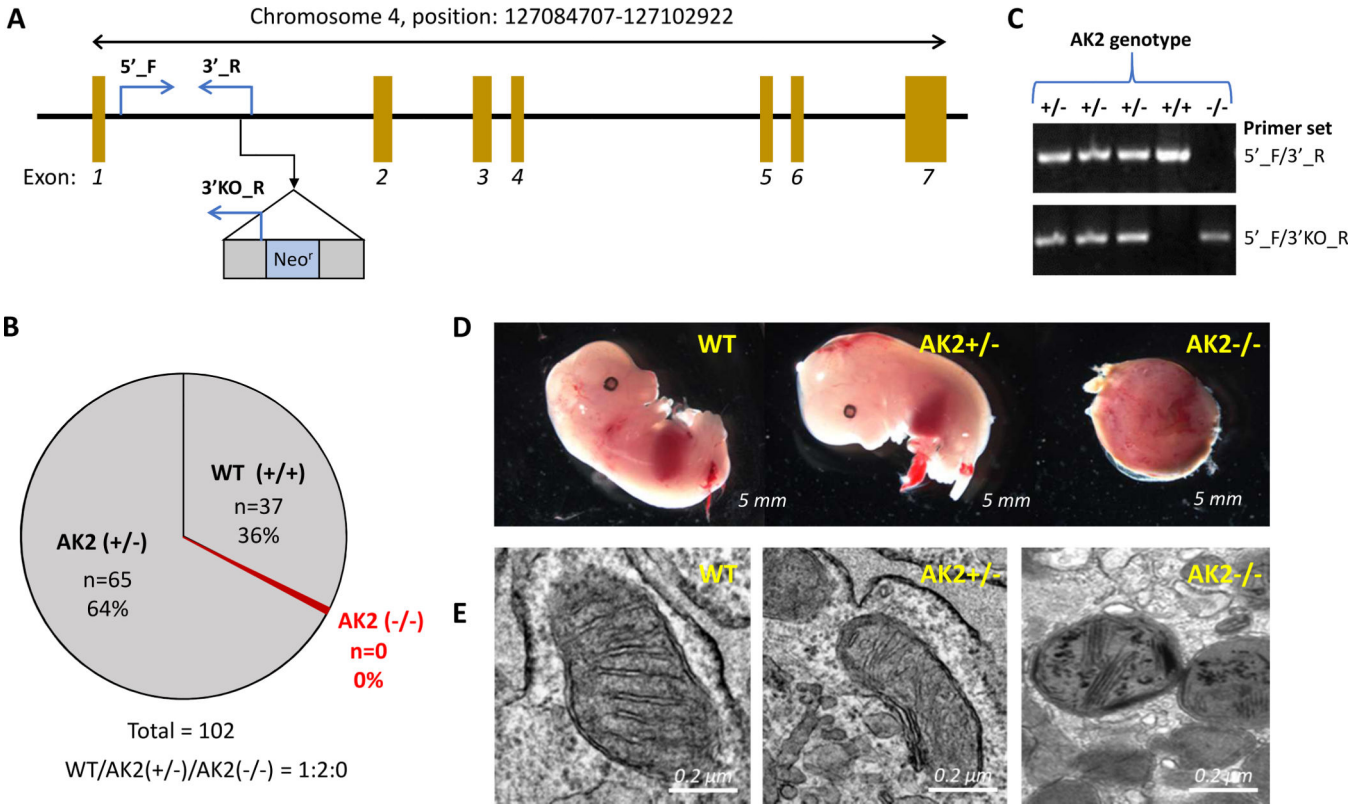

Figure 1.

AK2 germline gene deletion causes embryonic lethality. (A) AK2 gene trap mice were generated using the Omni Bank embryonic stem cell clone OST495245, which harbors the gene trapping vector VICTR CREATR 628 within the first intron of *AK2.* (B) Breeding of AK2+/− with (X) AK2+/− mice produced a wild-type (WT):AK2+/− offspring ratio of ~1:2 with no survival among AK2−/− homozygotes. (C) Genotyping indicates the absence of AK2 transcripts in AK2−/− embryos. (D, E) AK2 homozygous deletion caused embryonic lethality around day seven (E7.5) with severely disrupted mitochondria and cristae ultrastructure in AK2−/− embryonic tissue. In contrast, WT and AK2+/− heterozygous embryos developed normally.

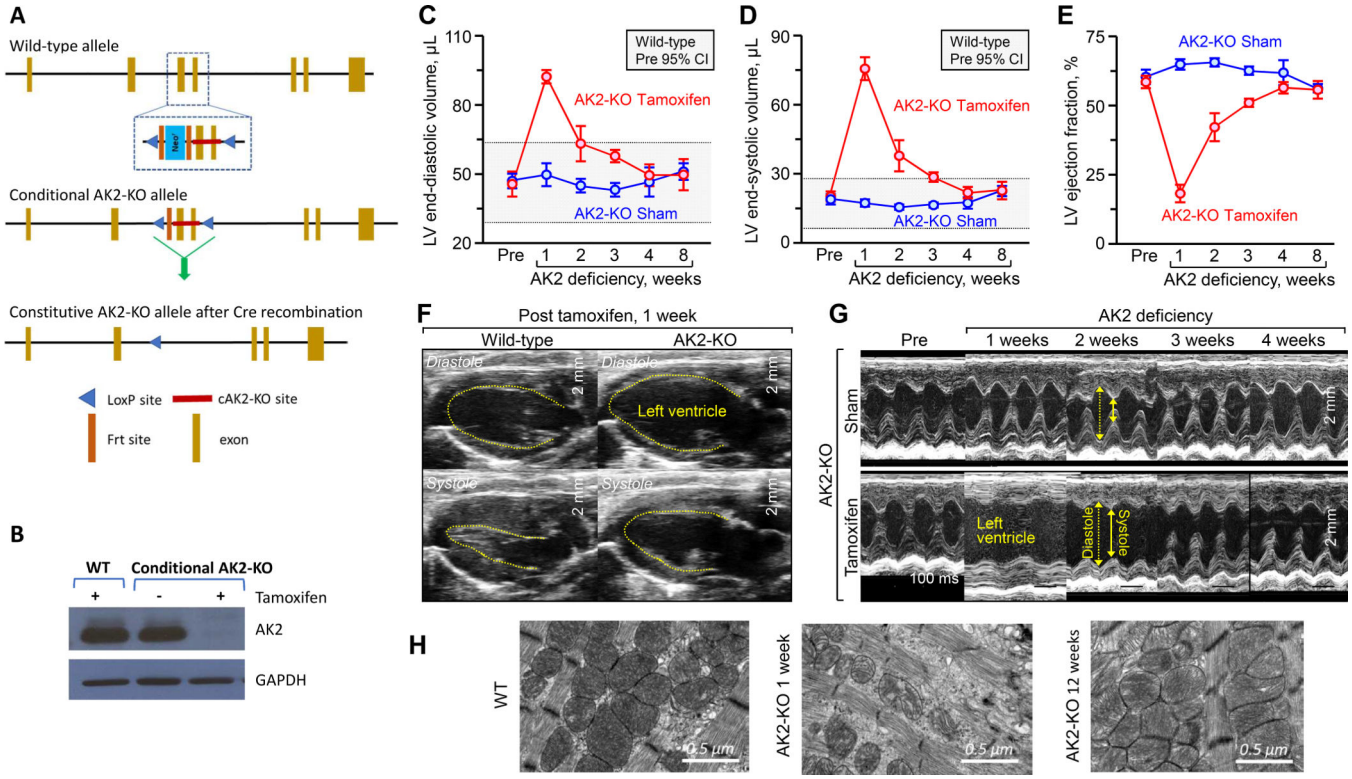

Figure 2.

Cardiac specific AK2 deletion precipitates abrupt heart failure with follow-up recovery. (A) Conditional heart-specific transgenic AK2 mice (AK2-KO) were engineered by deleting *AK2* exon 3 and 4 using Cre-Lox recombinase technology. (B) Western blot confirmed the absence of AK2 protein expression in tamoxifen-induced AK2−/− adult hearts. (C, D, E) Cardiac AK2−/− deficiency (AK2-KO Tamoxifen) provoked rapid organ deterioration with a pathological increase in end-diastolic and end-systolic volumes and decline in left ventricular ejection fraction within 1 week post-tamoxifen induction, which remarkably recovered by 12 weeks to pre-induction levels. (F, G) Echocardiography contrasting tamoxifen-induced AK2−/− from tamoxifen-induced WT and non-induced AK2−/− sham controls. CI, confidence interval; Sham, ethanol plus corn oil vehicle mixture. (H) Electron microscopic evaluation of hearts with conditional cardiac AK2−/− deletion 1 and 12 weeks after tamoxifen-induction.

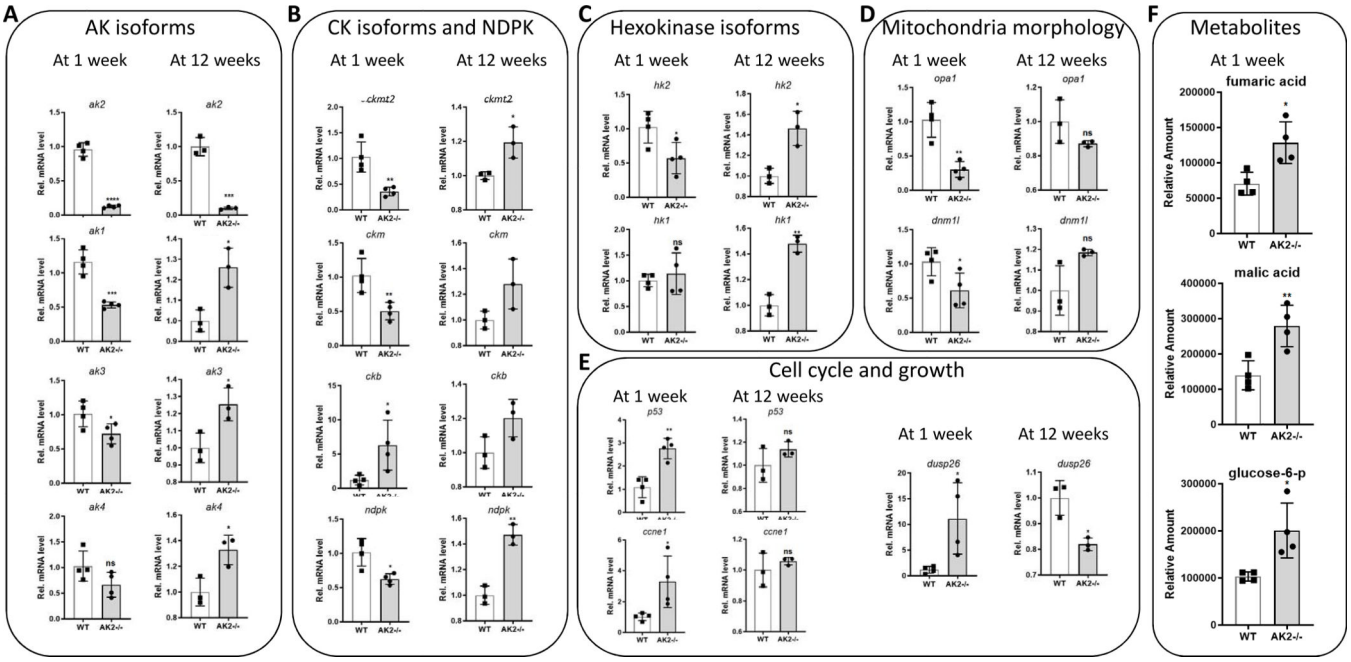

Figure 3.

Adaptive gene remodeling response characterizes the AK2 depleted state. (A, B, C) AK2 deletion depressed AK genes (*ak1*, *ak3* and *ak4*), creatine kinase genes (*ckmt2* and *ckm*), nucleotide diphosphate kinase gene (*ndpk*), and hexokinase gene (*hk2*) at 1-week post-tamoxifen induction. By 12 weeks, recovery of compromised gene expression followed. (D) Mitochondrial morphology genes (*opa1* and *dnm1*) also decreased transiently upon AK2 deletion, with recovery matching WT levels on follow-up. (E) Cell cycle and growth genes (*p53*, *ccne1* and *dusp26*) documented a broader gene remodeling pattern within the AK2 deficient heart. (F) Krebs cycle metabolites, fumarate and malate, along with the glycolytic metabolite, glucose-6-phosphate, accumulated in the AK2 deficient myocardium.