Characterization of Arginylation Branch of N-end Rule Pathway in G-protein-mediated Proliferation and Signaling of Cardiomyocytes

Min Jae Lee, Dong Eun Kim, Adriana Zakrzewska, Young Dong Yoo, Su-Hyeon Kim, Sung Tae Kim, Jai Wha Seo, Young Sook Lee, Gerald W. Dorn II, Uhm Tae Oh, Bo Yeon Kim, and Yong Tae Kwon

Department of Cell and Regenerative Biology, School of Medicine and Public Health, University of Wisconsin, Madison, Wisconsin 53706, and Department of Pharmacogenetics, Washington University School of Medicine, St. Louis, Missouri 63110

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Background: ATE1 transfers Arg to protein N termini, generating the degron for the N-end rule pathway.

Results: ATE1-deficient cardiomyocytes are impaired in the PLC/PKC-MEK1-ERK axis of Goq-mediated cardiac signaling.

Conclusion: The arginine branch of the N-end rule pathway controls G-protein signaling in cardiomyocytes in part through hypoxia-sensitive degradation of GAP proteins.

Significance: This study provides a cellular mechanism underlying cardiovascular defects observed in ATE1-deficient mice.

The N-end rule pathway is a proteolytic system in which destabilizing N-terminal amino acids of short lived proteins are recognized by recognition components (N-recogins) as an essential element of degrons, called N-degrons. In eukaryotes, the major way to generate N-degrons is through arginylation by ATE1 arginyl-tRNA-protein transferases, which transfer Arg from aminoacyl-tRNA to N-terminal Asp and Glu (and Cys as well in mammals). We have shown previously that ATE1-deficient mice die during embryogenesis with defects in cardiac and vascular development. Here, we characterized the arginylation-dependent N-end rule pathway in cardiomyocytes. Our results suggest that the cardiac and vascular defects in ATE1-deficient embryos are independent from each other and cell-autonomous. ATE1-deficient myocardium and cardiomyocytes therein, but not non-cardiomyocytes, showed reduced DNA synthesis and mitotic activity ~24 h before the onset of cardiac and vascular defects at embryonic day 12.5 associated with the impairment in the phospholipase C/PKC-MEK1-ERK axis of Goq-mediated cardiac signaling pathways. Cardiac overexpression of Goq rescued ATE1-deficient embryos from thin myocardium and ventricular septal defect but not from vascular defects.

The N-end rule pathway is a proteolytic system in which destabilizing N-terminal residues of short lived proteins function as an essential degradation determinant (1–4) (supplemental Fig. 1). Posttranslational conjugation of Arg to N-terminal Asp and Glu is a universal eukaryotic protein modification that generates the principal degron Arg (5–7). The arginylation branch of the N-end rule pathway is catalyzed by evolutionarily conserved arginyl-tRNA-protein transferase (ATE1 or R-transferase), which transfers Arg from Arg-tRNA to the acceptor residue Asp or Glu (6, 8). An acceptor substrate (Asp or Glu) of R-transferase can be exposed through a proteolytic cleavage of an otherwise stable polypeptide or deamidation of the pro-N-degron Asn or Gln by a specific N-terminal amidohydrolase (for review, see Refs. 3 and 9). In mammals, N-terminal Cys can also be converted to an arginylation-permissive pro-N-degron through a redox modification involving its oxidation into Cys sulfonic acid (CysO2(H)) or Cys sulfonic acid (CysO3(H)).
(10–12) (supplemental Fig. 1). N-terminal Arg together with other primary destabilizing residues (Lys, His, Phe, Tyr, Trp, Leu, and Ile) is recognized by a family of N-recognition (UBR1, UBR2, UBR4, and UBR5 in mammals) that promote N-degron-based polyubiquitylation and subsequent proteolysis through the 26 S proteasome (13, 14) (supplemental Fig. 1). Mammalian N-recognition share the UBR box, a zinc finger domain that binds preferentially the N-terminal Arg with a dissociation constant of low μM (15). The physiological functions and mechanisms of the N-end rule pathway are reviewed in Ref. 3.

The mammalian ATE1 gene produces at least six R-transferase isoforms, including those containing either of two homologous exons, through alternative splicing of pre-mRNAs (6). Although posttranslational arginylation was reported half a century ago (5), its physiological function has remained unclear until the discovery that knock-out of ATE1 in mice resulted in embryonic death (7). ATE1-deficient embryos die at embryonic day 15.5 (E15.5)–E16.5 with defects in cardiac and vascular development. Phenotypes of ATE1<sup>−/−</sup> hearts include ventricular myocardial hypoplasia associated with disorganized ventricular trabeculation, ventricular septal defect (VSD), and an outflow tract defect called persistent truncus arteriosus (PTA; alternatively called common arterial trunk) in which the truncus arteriosus is not properly separated into the pulmonary artery and aorta. ATE1<sup>−/−</sup> embryos also exhibit frequent hemorrhages and defective remodeling and branching of small vessels. Although these results suggest that ATE1 is required for development of embryonic hearts and maturation and/or integrity of blood vessels, the cellular function of arginylation in the cardiovascular lineage remains unknown. In addition to cardiovascular development, genetic analyses in mice implicated ATE1 in spermatogenesis (16, 17), metabolic homeostasis (16), and migration of neural crest cells (18). In the plant <i>Saccharomyces cerevisiae</i>, Ate1, the only R-transferase of the yeast N-end rule (15). The physiological functions and mechanisms of the N-end rule pathway are reviewed in Ref. 3.

**Experimental Procedures**

**Experimental Animals**—ATE1<sup>−/−</sup> mice were described in Ref. 7. ATE1 was inactivated by replacing exons 1 through 3 with the NLS-lacZ marker (β-galactosidase N-terminally fused with a nuclear localization signal) in C57 embryonic stem (ES) cells (7). ATE1<sup>−/−</sup> embryos were produced through heterozygous crosses in a 129SvJ/C57BL/6 background. Genotyping was carried out by using polymerase chain reaction (PCR) with primers F1 (CCAGCTCATTTCCCAACTGATCT), R1 (GGTATTTTGCTGCGCTTGTGGTGC), and R2 (CTG-GAGACAAAAAGCCCCAGCCAGAC), which amplify 570- and 430-bp fragments for wild type and knock-out alleles, respectively. ATE1<sup>−/−</sup>;MHC-Gα<sub>q</sub>40 mice were generated by mating ATE1<sup>−/−</sup> mice with MHC-Gα<sub>q</sub>40 transgenic mice (29), which express 40 copies of Gα<sub>q</sub> transgene in the heart from α-myosin heavy chain (MHC) promoter. Animal studies were conducted according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication Number 85-23, revised in 1996) and the protocols (0812811-A1) approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Euthanization involved inhalant anesthetic (isoflurane) followed by intraperitoneal injection of a xylazine (10 mg/kg) and ketamine (100 mg/kg) mixture.

**Primary Cardiomyocytes and Explanted Hearts**—Primary cardiomyocytes from mouse embryonic hearts were isolated as described with some modifications (30). Briefly, dissected hearts at E13.5 were digested in Hanks’ balanced salt solution containing 0.2% collagenase II, 0.005% trypsin, and 0.1% chicken serum for 15 min at 37 °C. The enzymes were inactivated with horse serum, and the cells were settled down by centrifugation and plated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Twenty-four hours after plating, the media were replaced by serum-free DMEM supplemented with 10 μg/ml insulin, 5.5 μg/ml transferrin, 5 μg/ml selenium, and 110 μg/ml pyruvate or with DMEM containing 10% horse serum and 5% FBS. Approximately 50% of the cells were determined to be cardiomyocytes by immunostaining with anti-sarcomeric α-actinin or anti-troponin I antibody (Santa Cruz Biotechnology). To culture embryonic hearts <i>ex vivo</i>, the hearts from E13.5 embryos containing outflow regions were incubated in DMEM containing 5% FBS, penicillin, and streptomycin, and the media were changed with serum-free DMEM containing supplements. The explanted hearts continued beating during incubation with 40 μM 5-bromo-2-deoxyuridine (BrdU) for 24 h in the presence or absence of agonists of G-protein-coupled receptor (GPCR). Proliferation of the hearts was examined by immunostaining BrdU on paraffin sections.

**Histology and β-Galactosidase Staining**—For histological analysis, embryos were fixed overnight at 4 °C in 4% paraformaldehyde (Fisher Scientific) in phosphate-buffered saline...
Cardiomyocytes of ATE1-deficient Embryos Are Impaired in Proliferation—To determine the cellular function of ATE1 in cardiac development, we observed the gross morphology of ~1,000 embryos at E10.5—E17.5 from ATE1<sup>−/−</sup> parents in a B6/129S background (Table 1). ATE1<sup>−/−</sup> embryos normally grew until E11.5 but began to show defects in cardiac and vascular development at E12.5 apparently without other morphological defects outside the cardiovascular system. ATE1<sup>−/−</sup> embryos at E12.5 showed VSD and thin myocardium in both ventricles associated with defective circulation as evidenced by pale yolk sacs (supplemental Figs. 2 and 3). By E13.5, the mutant embryos showed additional cardiovascular phenotypes observed in the previous study (7), including atrial septal defect, poorly developed trabeculae, dilated atria, PTA, and various vascular defects (local hemorrhages, poorly branched and thin-
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TABLE 1
Genotyping of embryos from intercrossing between ATE1+/− mice

| Age   | +/+ | +/− | −/− | −/− |
|-------|-----|-----|-----|-----|
| E0.5  | 12  | 21  | 14  | 14  |
| E1.5  | 31  | 63  | 25  | 25  |
| E2.5  | 29  | 52  | 24  | 24  |
| E3.5  | 71  | 133 | 77  | 77  |
| E4.5  | 50  | 78  | 45  | 45  |
| E5.5  | 17  | 32  | 9   | 9   |
| E6.5  | 10  | 18  | 5   | 5   |
| E7.5  | 4   | 46  | 11  | 11  |
| Postnatal | 223 | 404 | 0   | 0   |

* Found dead.

Significant difference between transferase dUTP nick end labeling) assays did not reveal a significant prolifeation rate is unlikely due to nonspecific growth arrest as lungs at E11.5 were used for a BrdU incorporation assay. Communostaining of BrdU and troponin I, a marker of cardiomyocytes, revealed a reduced proliferation in ATE1−/− cardiomyocytes (19.4% in +/+ versus 9.4% in −/−) (Fig. 2, A and B). In contrast, the difference was not obvious in troponin I-negative cardiac cells, which are mainly composed of cardiac fibroblasts with a minor contribu- tion by endothelial cells and smooth muscle cells (62.3% in +/+; 58.1% in +/−; 60.9% in −/−) (Fig. 2B). An analogous assay with an antibody to phosphorylated histone H3 also sug- gested that the mitotic activity of cardiomyocytes is significantly reduced in the absence of ATE1 (supplemental Fig. 5).

To determine whether ATE1 is expressed in cardiomyocytes, cultured primary cardiac cells from E13.5 ATE1+/− embryos were subjected to enzymatic staining for the reporter NLS-lacZ, which marks the ATG codon of ATE1. Immunostaining of sarcomeric α-actinin, a marker of cardiomyocytes, following lacZ staining revealed a robust expression of ATE1 in cardiomyocytes (Fig. 2C). By contrast, the expression of ATE1 was much lower or often undetectable in α-actinin-negative non-cardiomyocytes. These results suggest that ATE1 knock-out results in significantly reduced proliferation in cardiomyocytes without severe defects in the developmental program or increased apoptosis.

ATE1-deficient Embryonic Hearts Are Impaired in G-protein Signaling—Gene mutations associated with cardiac defects have been implicated in signaling molecules, cell adhesion molecules, ion channels, and transcription factors (33). To test a potential function of arginylation in extracellular signaling pathways of cardiomyocytes, a BrdU incorporation assay was performed on primary cardiac cells from +/+ and ATE1−/− embryos at E13.5 before and after the treatment with ligands to GPCRs and receptor tyrosine kinases, including prostaglandin F2α (for prostaglandin F receptor coupled with Gq), phenylephrine (for α-adrenergic receptor coupled with Gq and Gβγ), basic FGF (for fibroblast growth factor basic receptor, a receptor tyrosine kinase), isoproterenol (for β-adrenergic receptor coupled with Gs), and angiotensin II (for AT1 receptor coupled with Gq and Gβγ). Among these, only angiotensin II significantly promoted the proliferation of troponin I-positive cardiomyocytes (Fig. 3, A and B). Notably, ATE1−/− cardiomyocytes failed to properly respond to angiotensin II compared with +/+ cells. To determine angiotensin II-induced proliferation in a more physiological condition, we used an ex vivo model for +/+ and ATE1−/− hearts from E13.5 embryos. An analogous assay on explanted hearts showed that ATE1−/− hearts are impaired in angiotensin II-induced proliferation (Fig. 3C and supplemental Fig. 6A). The mRNA level of the AT1 receptor was comparable in cultured cardiac cells from +/+ and ATE1−/− hearts as determined by quantitative RT-PCR (supplemental Fig. 6B).

Extrinsic stimuli such as endothelin-1, angiotensin II, and phenylephrine induce cell growth in the heart through their interaction with GPCRs that activate the Gq class of GTP-binding proteins (34). Upon binding to an agonist-occupied receptor, the heterotrimeric Gq protein dissociates into individual Gaq and Gβγ subunits. GTP-bound Gaq activates PLC, which results in inositol 1,4,5-trisphosphate-mediated calcium release and diacylglycerol-mediated activation of PKC. Dissoc- iated Gβγ has the potential to activate the small GTP-binding protein Ras and initiate a tyrosine kinase cascade, leading to activation of MAPKs. Gaq can also activate MAPKs independ- ently from Gβγ via a PKC-dependent mechanism. To determine the function of ATE1 in Gq signaling of cardiomyocytes, we measured the enzymatic activities of signaling molecules in extracts of +/+ and ATE1−/− embryonic hearts at E13.5.

Other small vessels, irregularly terminated large vessels, and pale yolk sacs (supplemental Figs. 2 and 3). Notably, all of the ATE1−/− hearts with PTA (n = 10) also contained VSD, whereas only ~50% of the hearts with VSD (n = 19) exhibited PTA, suggesting that VSD and PTA in ATE1−/− hearts may involve misregulation in two independent processes, for example myocardial migration and proliferation of neural crest cells, respectively. No live animals were retrieved beyond E15.5. Despite defective cardiogenesis, the expression of the following markers involved in cardiac development was comparable in E13.5 ATE1−/− hearts as determined by quantitative RT-PCR: GATA4, Nkx2.5, MEF2C, DHAN, eHAND, NPPA, β-miosin heavy chain, cardiac α-actin, skeletal α-actin, Srf, and Atp2a2 (data not shown). Thus, the cardiac defects are not mainly due to misregulation of developmental processes at the transcriptional level.

To determine the proliferation of ATE1−/− embryonic hearts, we intraperitoneally injected BrdU into pregnant females, harvested embryos at different stages, and performed immunofluorescence staining of BrdU on the cross-sections of embryos. The hearts of E12.5 ATE1−/− embryos exhibited reduced levels of S phase cells in ventricular walls (26% in +/+ versus 12% in −/−) and intraventricular septum (33% in +/+ versus 10.4% in −/−) (Fig. 1, A and B). The reduced DNA synthesis rate is unlikely due to nonspecific growth arrest as lungs of the same mutant embryos showed a normal S phase index (57% in +/+; 60.2% in −/−) (Fig. 1C). As an independent measurement, we performed an analogous assay with an antibody specific to phosphorylated histone H3, a hallmark of mitosis. The hearts of E11.5 ATE1−/− embryos exhibited reduced levels of M phase cells in ventricular walls (1.8% in +/+; 0.52% in −/−), ventricular septum (1.4% in +/+; 0.73% in −/−), and trabeculae (1.5% in +/+; 0.72% in −/−) (supplemental Fig. 4). The reduced proliferation is not because more cardiac cells are eliminated by apoptosis as TUNEL (terminal deoxynucleotidyltransferase dUTP nick end labeling) assays did not reveal a significant difference between +/+ and ATE1−/− hearts at E11.5 through E12.5 (data not shown).

To determine whether arginylation is required for the proliferation of cultured cardiomyocytes, primary cardiac cells established from +/+ and ATE1−/− embryos at E13.5 were used for a BrdU incorporation assay. Communostaining of BrdU and troponin I, a marker of cardiomyocytes, revealed a reduced proliferation in ATE1−/− cardiomyocytes (19.4% in +/+ versus 9.4% in −/−) (Fig. 2, A and B). In contrast, the difference was not obvious in troponin I-negative cardiac cells, which are mainly composed of cardiac fibroblasts with a minor contribu-
ATE1/−/− hearts contained reduced activities for several enzymes that mediate Goq signaling, such as PKC (Fig. 4A) and PLC (supplemental Fig. 7A). By contrast, no difference was observed for PKA (Fig. 4B) and Ca2+/calmodulin-dependent protein kinase II (supplemental Fig. 7B), which are activated by Gq-dependent adenyl cyclase. PLC and PKC activate Gq-S progression through the MAPK pathway. To determine a specific MAPK subpathway linked to ATE1-dependent arginylation, we monitored the kinetics of activation and inactivation of candidate components in primary cardiac cells established from +/+ and ATE1/−/− embryonic hearts at E13.5. Time course immunoblotting following 24-h serum starvation and subsequent serum activation identified MAPK/ERK kinase 1 (MEK1) as a component whose activity is markedly attenuated in ATE1/−/− cardiomyocytes (Fig. 4C). An analogous assay for MAPKs showed that the activities of ERK1 and ERK2, which are phosphorylated by MEK1, were significantly down-regulated in ATE1/−/− hearts (Fig. 4C), which was verified by the immunohistochemistry analysis (supplemental Fig. 7C) and an in vitro kinase assay (supplemental Fig. 7D).

The MAPK pathway can induce Gq-S progression through transcriptional induction of cyclin A, which binds to cyclin-dependent kinase 2. To determine the effect of ATE1 knock-out on the activation of cyclins, primary cardiac cells established from +/+ and ATE1/−/− embryonic hearts at E13.5 were subjected to serum stimulation following 24-h serum starvation. Immunoblotting analysis revealed a robust induction for cyclin A in ATE1+/+ cells that was markedly diminished in mutants (Fig. 4D, bottom). Under these conditions, no significant differences were observed for cyclins H and D3. As an alternative approach, we used fluorescence-activated cell sorting (FACS) analysis using cultured cardiac cells at passage number 5 (to obtain a sufficient number of cells) that were derived from +/+ and ATE1/−/− embryonic hearts at E13.5. The percentage of ATE1/−/− cells in S phase (16.3% in +/+ versus 5.4% in −/−), but not GoqG12a phase (61.7% in +/+ versus 78.3% in −/−), was significantly lower compared with controls. These results together suggest that cardiovascular defects of ATE1/−/− embryos are in part contributed by misregulation of the Goq signaling PLC/PKC-MEK1-ERK1 axis of G-protein signaling in embryonic hearts.

Cardiac Overexpression of Goq Significantly Rescues ATE1-deficient Mouse Embryos from Ventricular Septal Defects and Thin Myocardium—To determine whether cardiac overexpression of Goq improves cardiac development in ATE1/−/− embryos, we generated a double mutant strain (ATE1/−/−; MHC-Goq40) by crossing ATE1/−/− mice in a C57BL/6J-129SvEv background with MHC-Goq40 transgenic mice in an FVB/N background. It has been shown that MHC-Goq40 transgenic mice (see “Experimental Procedures”) develop cardiovascular hypertrophy in adulthood associated with induction of fetal gene expression and reduced cardiac contractility (29). Immuno blotting analysis showed a 5-fold overexpression of Goq in the hearts of ATE1/−/−;MHC-Goq40 embryos at E15.5 compared with littermate controls (Fig. 5A), whereas no differences were observed for the liver, lung, and brain (Fig. 5B).

Cardiac overexpression of Goq did not cause a significant difference in the gross morphology of ATE1/−/− and ATE1/−/−; MHC-Goq40 embryos when observed at E14.6 through E16.5 (Fig. 5, C and D). In both genotypes, local hemorrhages indicative of circulation defects were obvious at E14.5 and became severe at E15.5 through E16.5. Overall, the morphological phenotypes (Fig. 5, C and D) observed in ATE1/−/− and ATE1/−/−; MHC-Goq40 embryos in the C57/129/FVB background were indistinguishable from those of embryos in the C57/129 background that have been characterized in the previous (7) and
current (supplemental Figs. 2 and 3) studies. Importantly, when embryonic hearts were harvested and morphologically examined, cardiac overexpression of Gαq did rescue significantly ATE1−/− hearts from cardiac defects. For instance, in sharp contrast to E16.5 ATE1−/− hearts (n = 5) morphologically arrested at ~E14.5 (Fig. 6, C versus A), ATE1−/−;MHC-Gαq40 hearts (n = 8) at the same stage showed virtually normal morphology relative to wild-type and MHC-Gαq40 embryos (Fig. 6, D versus A and B). In addition, cross-sections of E16.5 ATE1−/−;MHC-Gαq40 hearts showed significant rescue effects for thin myocardium, VSD, trabeculation, and atrial septal defect (Fig. 6D and data not shown) relative to control ATE1−/− hearts. These results suggest that overexpression of Gαq in the heart significantly rescues ATE1−/− embryos from cardiac defects.

**Vascular Defects in ATE1-deficient Mouse Embryos Are Independent from Cardiac Defects**—Despite cardiac rescue by Gαq overexpression, ATE1−/−;MHC-Gαq40 embryos still died around E15.5 and E16.5 with no obvious difference in timing and morphology compared with control ATE1−/− embryos, suggesting that cardiac defects are not the primary cause of death in ATE1-deficient embryos. To determine whether vascular defects observed in ATE1−/− embryos are independent from cardiac defects, we examined the gross morphology of embryos at E14.5 through E16.5. ATE1−/−;MHC-Gαq40 embryos (n = 16) invariably developed morphological defects indistinguishable from vascular defects in the ATE1−/− yolk sac and embryos proper observed in a previous (7) and this (n = 15) study (Fig. 5, C and D). Although we do not exclude the possibility of subtle changes in vascular integrity, these results suggest that vascular defects in ATE1−/− embryos may be the primary cause of death and independent from cardiac defects.

**FIGURE 2.** Primary cardiomyocytes from ATE1-deficient embryos at E13.5 are impaired in proliferation. A, BrdU incorporation assay of primary cardiac cells derived from +/+ and ATE1−/− embryonic hearts at E13.5. Cardiomyocytes were identified by immunostaining of troponin I. Cardiomyocytes are distinguished from non-cardiomyocytes by immunostaining of troponin I or sarcomeric α-actin. Scale bars, 10 μm. B, quantitation of A, C, the enzymatic staining of β-galactosidase in primary cardiac cells from ATE1−/− embryos at E13.5. Data are presented as mean ± S.D.

**FIGURE 3.** Cardiomyocytes of ATE1-deficient embryos are impaired in angiotensin II-induced G-protein signaling. A, cultured primary cardiac cells from +/+ and ATE1−/− embryos at E13.5 were treated with 2 μM angiotensin II (AngII) and subjected to a BrdU incorporation assay with coimmunostaining of troponin I and MHC-Gαq40. Scale bar, 10 μm. B, quantitation of an analogous assay (A) in which cells were treated with various ligands to GPCR: 50 ng/ml basic fibroblast growth factor (FGFb), 2 μM angiotensin II (AngII), 100 nM prostaglandin F2α (PGF2α), 200 μM phenylephrine (PE), and 4 μM isoproterenol (ISO). C, explanted hearts from +/+ and ATE1−/− embryos at E13.5 were treated with 2 μM angiotensin II followed by a BrdU incorporation assay on cross-sections of the left ventricle (LV). Scale bar, 200 μm. Data are presented as mean ± S.D.
Degradation of RGS4 Spatiotemporally Correlates to ATE1 Distribution in Mouse Embryos and Is Sensitive to Oxygen Availability—The substrates of arginylation that may underlie cardiovascular defects in ATE1 \(^{-/-}\) embryos include a set of structurally related RGS proteins (RGS4, RGS5, and RGS16) that can act as GTPase-activating proteins for Goq (11, 12, 22). RGS4 and RGS5 have been characterized as regulators of G-protein signaling in the heart and blood vessels, respectively (35–38). In cultured cells, the degradation of RGS4 can be mediated by arginylation-dependent N-end rule ubiquitylation (11, 22) or an internal degron (non-N-degron)-based ubiquitylation by an unknown E3 ligase (data not shown) depending on cell types and states.

To determine the spatiotemporal relationship between RGS4 and ATE1, we performed whole-mount immunostaining of RGS4 in \(+/+\) and \(ATE1^{-/-}\) embryos at E12.5 and E13.5. RGS4 was barely detectable in \(+/+\) but was drastically accumulated in \(ATE1^{-/-}\) embryos (Fig. 7A and data not shown), indicating strong arginylation-dependent degradation of endogenous RGS4 in growing embryos. Immunoblotting analysis of whole embryos and embryonic hearts showed an accumulation of RGS4 in the absence of ATE1 (Fig. 7, B and C) without a significant change in transcription (supplemental Fig. 8A). Immunostaining on cross-sections revealed a strong correlation between RGS4 and ATE1 in all cell types examined that express RGS4 (data not shown), including hearts (supplemental Fig. 8B). RGS4-positive cells were relatively enriched along the migratory pathway of neural crest cells, including dorsal root ganglia, sympathetic ganglia, muscle lineage, and developing alveoli (supplemental Fig. 9). In these cells, the expression of ATE1 was also prominent (Ref. 7 and data not shown). Consistently, previous studies suggested that both RGS5 and ATE1 are prominently expressed in arteries (7, 38, 39). These results indicate that ATE1 plays a role in homeostasis of G-protein signaling in hearts and other tissues through regulated proteolysis of multiple RGS proteins. The rapid degradation of RGS4 also explains why its expression at the protein level currently remains elusive in the hearts even though an abundant mRNA expression correlates to cardiac proliferation and hypertrophy (40, 41).

RGS4 has the N-terminal Met-Cys sequence in which the Cys-2 residue is a degradation signal (degron) for the N-end rule pathway (11). We have observed previously that both RGS5 and ATE1 are prominently expressed in arteries (7, 38, 39). These results indicate that ATE1 plays a role in homeostasis of G-protein signaling in hearts and other tissues through regulated proteolysis of multiple RGS proteins. The rapid degradation of RGS4 also explains why its expression at the protein level currently remains elusive in the hearts even though an abundant mRNA expression correlates to cardiac proliferation and hypertrophy (40, 41).

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FIGURE 6. Overexpression of Goα subunit in heart improves cardiac development in ATE1−/− embryos. Shown are gross morphology (panels a), cross-sections (panels b), and close-up views of left ventricles (panels c) of embryonic hearts. Genotypes of these hearts are shown to the left: wild-type (A), MHC-Goα40 (B), ATE1−/− (C), and ATE1+/−;MHC-Goα40 (D) embryos. Scale bars, 300 (panels a and b) and 100 μm (panels c). RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle. Arrowheads in panel c indicate the thickness of left ventricular walls. Note that the ventricular wall of the ATE1−/−;MHC-Goα40 heart (D) is comparable with that in wild-type heart (A).

the previous observation (11), in normoxia, RGS4 was rapidly degraded with a drastically reduced level (~14%) of the zero time point compared with C2V-RGS4 in which a mutation of the pro-N-degron Cys-2 to Val inhibits arginylation (Fig. 7, D and E). Notably, in hypoxia, normally short lived RGS4 was significantly stabilized as compared with the half-lives of C2V-RGS4 in normoxia and hypoxia (Fig. 7, D and E). A previous study reported the hypoxia-sensitive degradation for a set of short lived proteins carrying the Met-Cys sequence in which a mutation of the pro-N-degron Cys-2 is constitutively degraded to a maximal level (Fig. 8). However, when O2 (or

DISCUSSION

In the current study, we studied the physiological function of the arginylation branch of the N-end rule pathway in embryonic hearts and cardiomyocytes. We report that thin myocardium and VSD of ATE1−/− embryonic hearts first observed at E12.5 is primarily caused by a specific impairment of proliferation in cardiomyocytes but not cardiac fibroblasts, consistent with the prominent expression of ATE1 in cardiomyocytes relative to cardiac fibroblasts. Our results suggest that ATE1-deficient hearts and cardiomyocytes therein, but not cardiac fibroblasts, are impaired in the PLC/PKC-MEK1-ERK axis of Goα-activated protein signaling. By overexpressing Goα in ATE1−/− hearts using the MHC promoter, we were able to rescue significantly ATE1−/− embryos from thin myocardium and VSD. Of note, cardiac overexpression of Goα did not noticeably affect vascular defects, demonstrating that cardiac and vascular defects of ATE1−/− embryos are largely independent from each other and cell-autonomous and that vascular defects may be the primary cause of death in ATE1−/− embryos. The impaired G-protein signaling is attributed in part to failure to mediate arginylation-dependent degradation of RGS4 (and RGS5 and RGS16 as well) known to function as a GTPase-activating protein for GPCR-activated Goα during cardiac G-protein signaling. Given the biochemical property and physiological function of RGS4 as a negative regulator of Goα in the heart models (35, 36), it is reasonable to speculate that abnormal accumulation of RGS4 (and RGS5 and RGS16 as well) impairs G-protein signaling in cardiomyocytes, contributing to the growth arrest of myocardium during embryogenesis. However, it should be noted that ATE1 has been implicated in a variety of physiological processes, including arginylation of many cellular proteins (see the Introduction). Therefore, there are likely to be additional molecular mechanisms that contribute to cardiovascular defects in ATE1−/− embryos.

The mammalian heart consumes 3–20 times more O2 than the brain (44) and thus requires a constant supply of O2 for its function. For example, coronary artery disease with consequent myocardial ischemia and necrosis is a leading cause of heart failure worldwide. Although O2 is a major determinant of cardiac gene expression and numerous cellular processes, little is known about its role in cardiovascular signaling and the mechanism by which the heart senses its concentration to modulate intracellular processes. Our results indicate hypoxia-sensitive, arginylation-dependent degradation of RGS4 with the finding that ATE1-marked β-galactosidase and RGS5 mRNAs are prominently expressed in artery relative to veins (7, 37–39, 45). In mouse embryonic fibroblasts, the degradation of RGS4 involves the cleavage of N-terminal Met, which exposes the pro-N-degron Cys at the N terminus (11) (Fig. 8). The exposed Cys-2 is conjugated with a mass of 48 Da that is thought to represent oxidation to CysO2(H) and subsequent conversion to CysO2H, whose structure is similar to the arginylation-permissive pro-N-degron Asp (7, 11, 12) (supplemental Fig. 1). Therefore, it is likely that the reduced availability of O2 inhibits the Cys-2 oxidation prior to arginylation by ATE1. These observations suggest that in hearts and blood vessels under normal physiological conditions in which cells are exposed to sufficient O2 and NO RGS4 with the pro-N-degron Cys-2 is constitutively degraded to maintain G-protein signaling, allowing cells to sense extracellular ligands to a maximal level (Fig. 8). However, when O2 (or
other molecules that induce Cys-2 oxidation in circulating blood is not sufficient, for example in ischemia caused by cardiac arrest or other cellular stresses, these substrates are rapidly accumulated in a real time basis to turn down GPCR signaling, decoupling cells from extracellular proliferation signals (Fig. 8). Thus, arginylation-induced proteolysis may function as a cellular stress response to maintain homeostasis in GPCR signaling in the heart (via RGS4) and blood vessels (via RGS5).

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