HOKKAIDO UNIVERSITY

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| Title | Tyrosine kinase FYN negatively regulates NOX4 in cardiac remodeling |
| Author(s) | Matsushima, Shouji; Kuroda, Junya; Zhai, Peiyong et al. |
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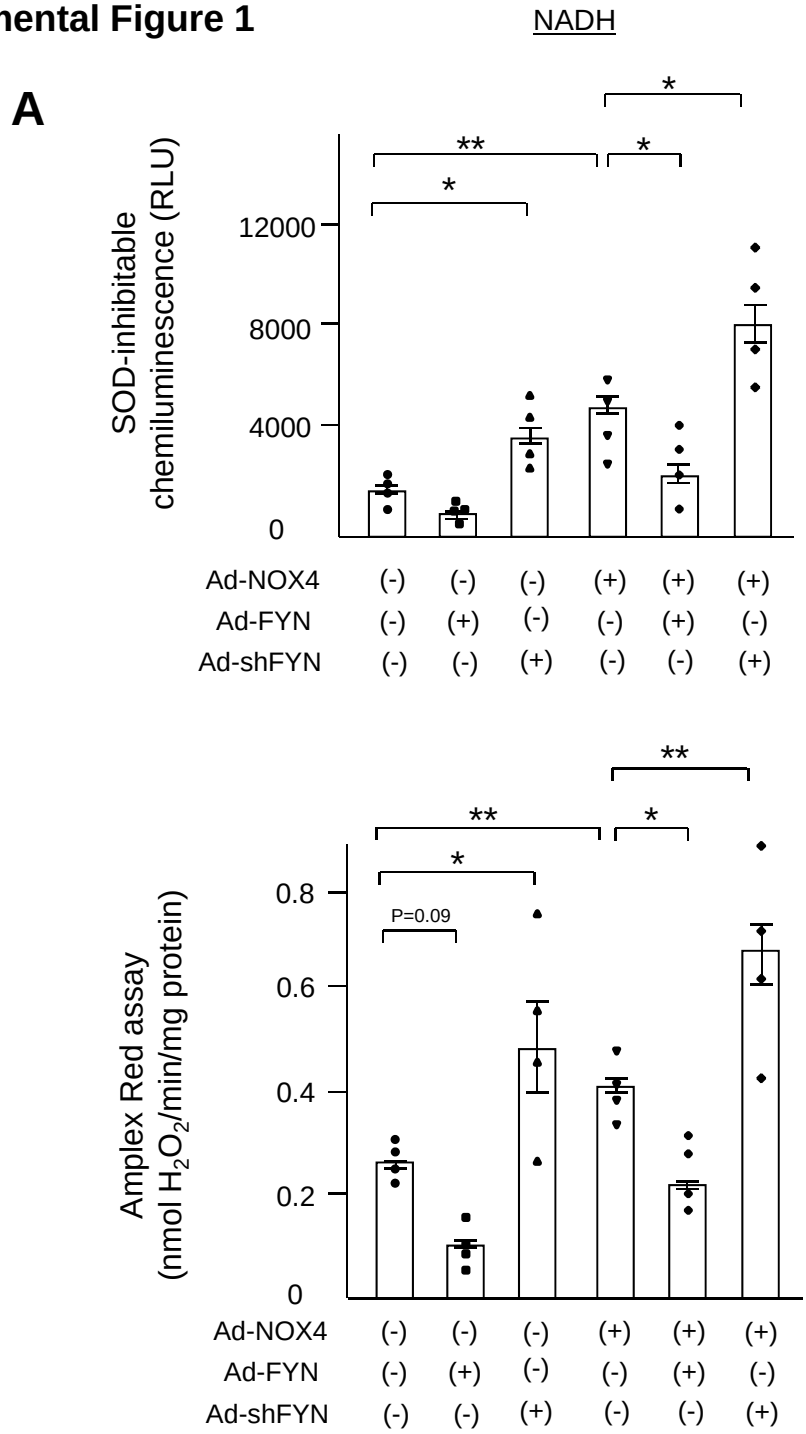
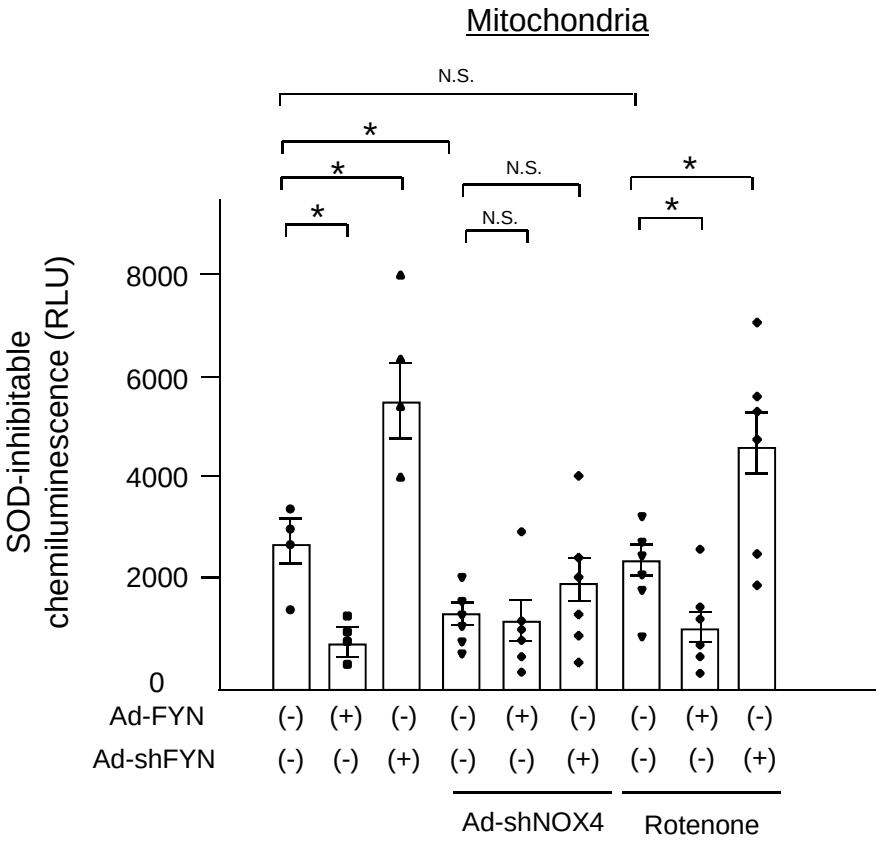
**Supplemental Figure 1**



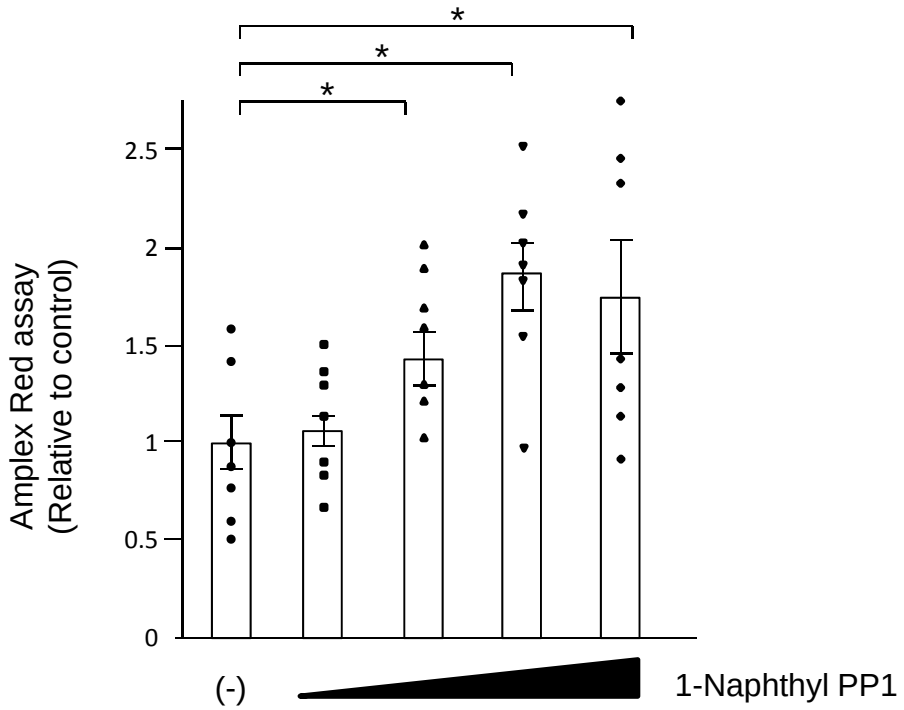

**Supplemental Figure 1 A.** NADH-dependent $O_2^-$ release was measured by the lucigenin method. The SOD-inhibitable component of $O_2^-$ release from the mitochondrial fraction in CMs transduced with indicated adenoviruses was determined (n=4). **B.** $H_2O_2$ content in mitochondria was measured by Amplex Red assay (n=4). Statistical analyses were done by one-way ANOVA followed by a post hoc Fisher's comparison test. *****P<0.05, ******P<0.01.

## Supplemental Figure 2



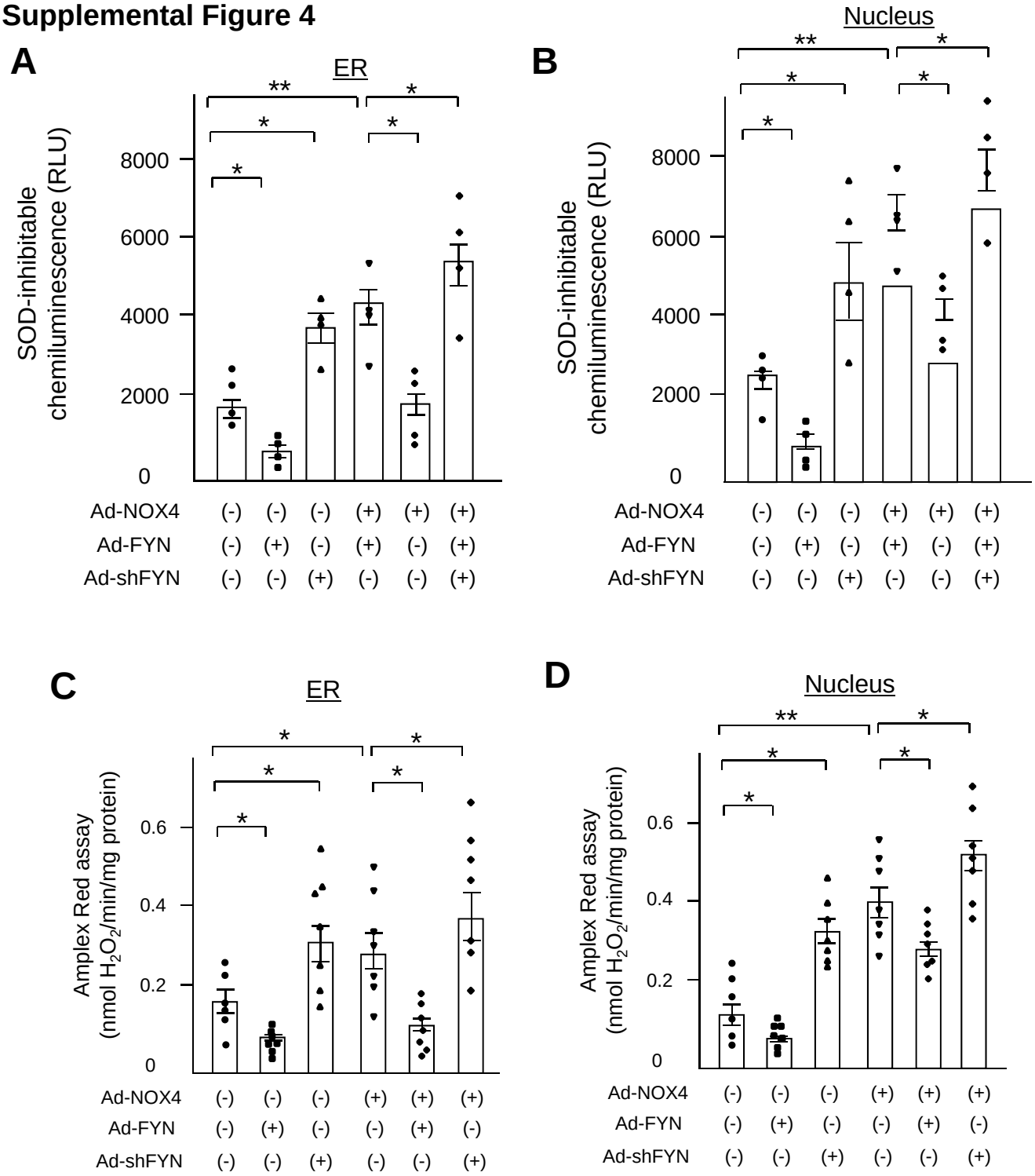

**Supplemental Figure 2** NADPH-dependent $O_2^-$ release was measured by the lucigenin method. The SOD-inhibitable component of $O_2^-$ release from the mitochondrial fraction in CMs transduced with indicated adenoviruses was determined (n=4-6). Rotenone (10 nM) was added to mitochondrial fractions 5 min before the addition of NADPH. Statistical analyses were done by one-way ANOVA followed by a post hoc Fisher's comparison test. *P<0.05.

## Supplemental Figure 3



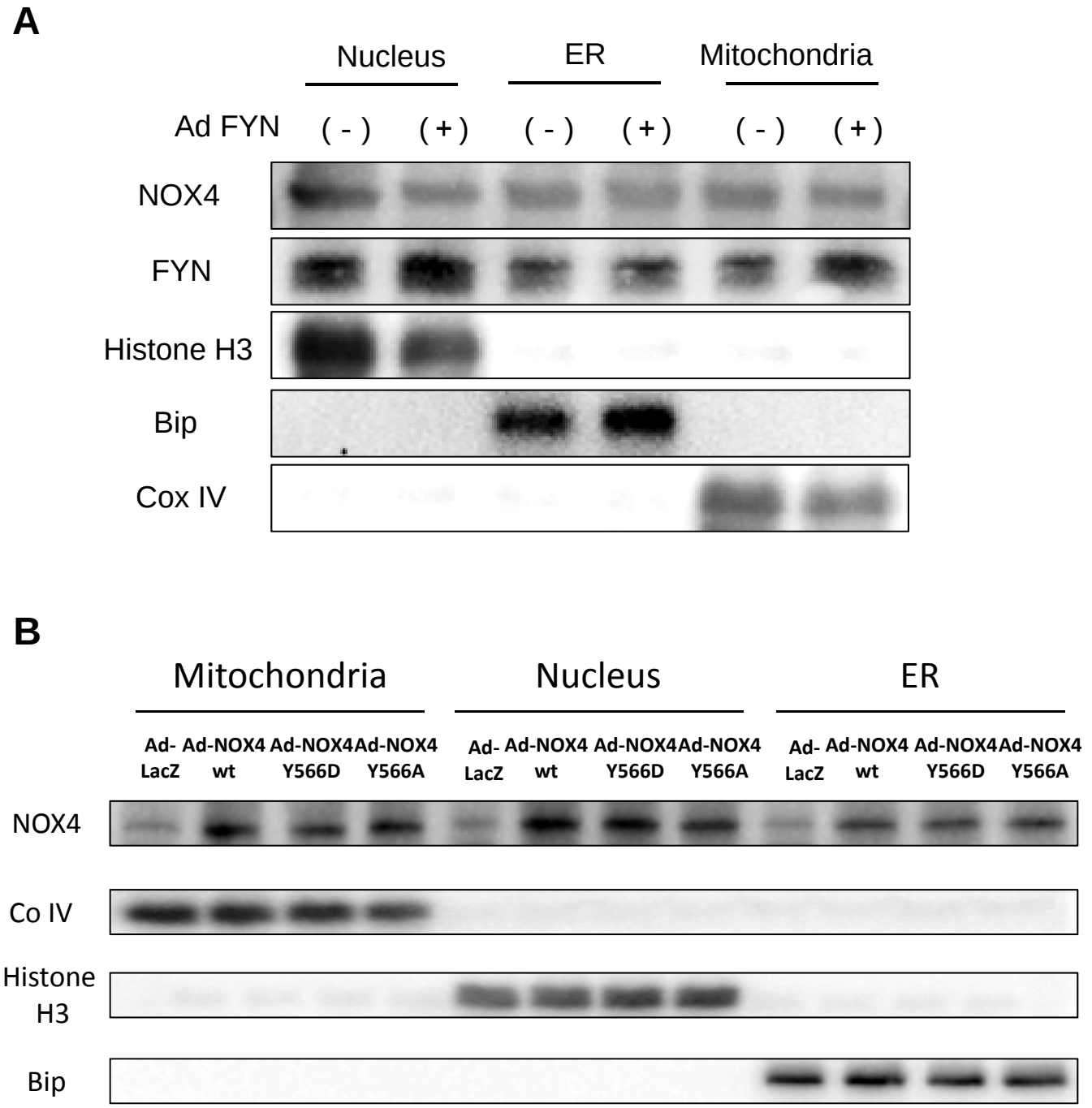

**Supplemental Figure 3** Cardiomyocytes were treated with a Fyn inhibitor (1-Naphthyl PP1, 0, 0.3, 1, 3, and 10 μM) for 30 minutes. $H_2O_2$ production was examined with Amplex Red assays (n=7). Statistical analyses were done by one-way ANOVA followed by a post hoc Fisher's comparison test. *****P<0.05.

**Supplemental Figure 4**



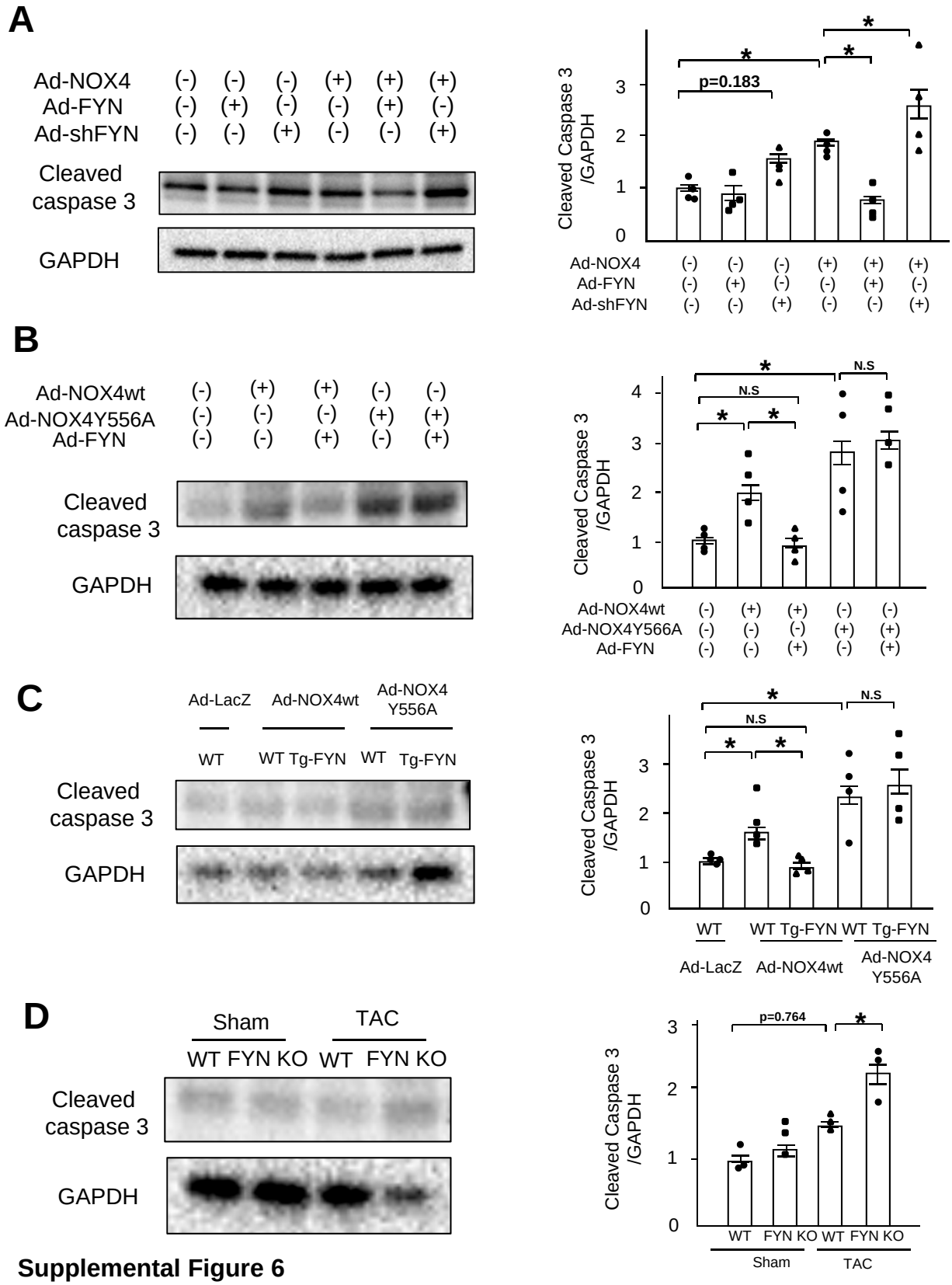

**Supplemental Figure 4** NADPH-dependent $O_2^-$ release was measured by the lucigenin method. **A, B.** The SOD-inhibitable component of $O_2^-$ release from ER fraction (**A**) and nuclear fraction (**B**) in CMs transduced with indicated adenoviruses was determined (n=4). **C, D.** $H_2O_2$ content in ER fraction (**C**) and nuclear fraction (**D**) was measured by Amplex Red assay (n=6-7). Statistical analyses were done by one-way ANOVA followed by a post hoc Fisher's comparison test. *****P<0.05, ******P<0.01

**Supplemental Figure 5**




**Supplemental Figure 5** **A.** Protein levels of NOX4, FYN, Histone H3, Bip, and COXIV in nucleus, ER, and mitochondria in CMs transduced with indicated adenovirus were determined. The experiment was conducted 3 times. **B.** Protein levels of NOX4, Histone H3, Bip, and COXIV in nucleus, ER, and mitochondria in CMs transduced with indicated adenovirus were determined. The experiment was conducted 3 times.

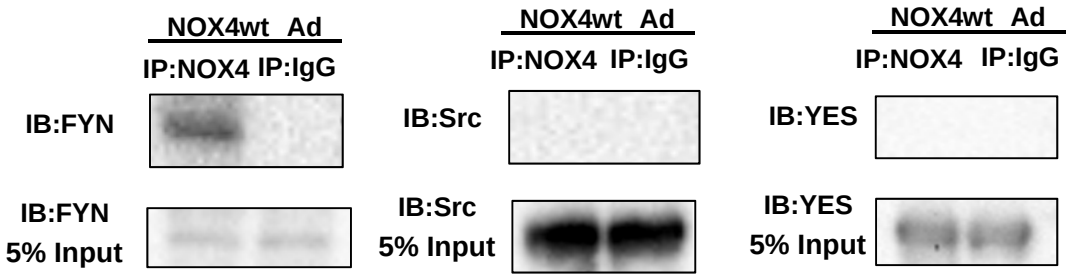
## Supplemental Figure 6




**Supplemental Figure 6**

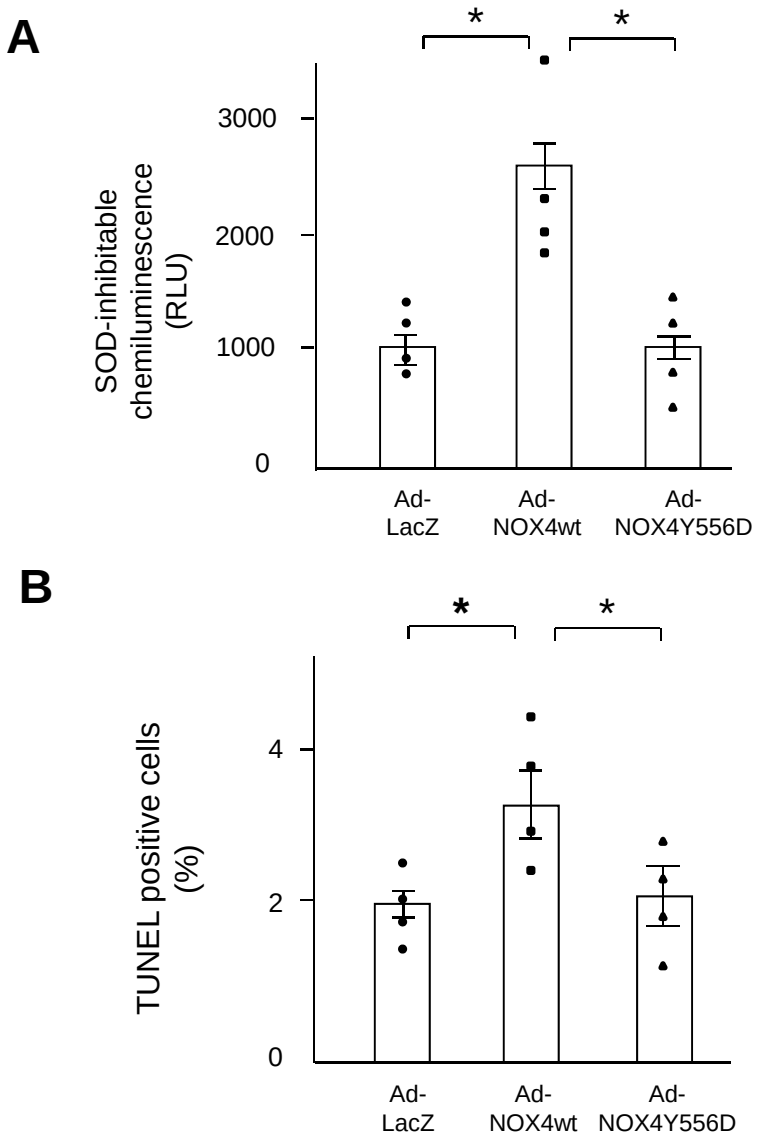
**A, B.** Protein levels of cleaved caspase3 and GAPDH in cultured neonatal rat CMs transduced with indicated adenoviruses. **C, D.** Protein levels of cleaved caspase3 and GAPDH in the heart from indicated mice. Statistical analyses were done by one-way ANOVA followed by a post hoc Fisher's comparison test. *****P<0.05

## Supplemental Figure 7




**Supplemental Figure 7**
Co-immunoprecipitation assays using lysates of CMs transduced with Ad-NOX4wt. After immunoprecipitation (IP) with control IgG or anti-NOX4 antibody, immunoblotting for FYN, Src, or YES was performed. Immunoblots of input controls (5% lysates) are also shown. The experiment was conducted 3 times.

## Supplemental Figure 8




**Supplemental Figure 8**
**A.** NADPH-dependent O2- release was measured by the lucigenin method. The SOD-inhibitable component of O2- release from the mitochondrial fraction in CMs transduced with indicated adenoviruses was determined (n=4). **B.** Apoptosis in CMs transduced with indicated adenoviruses was evaluated with TUNEL staining (n=4). Statistical analyses were done by one-way ANOVA followed by a post hoc Fisher's comparison test. *P<0.05

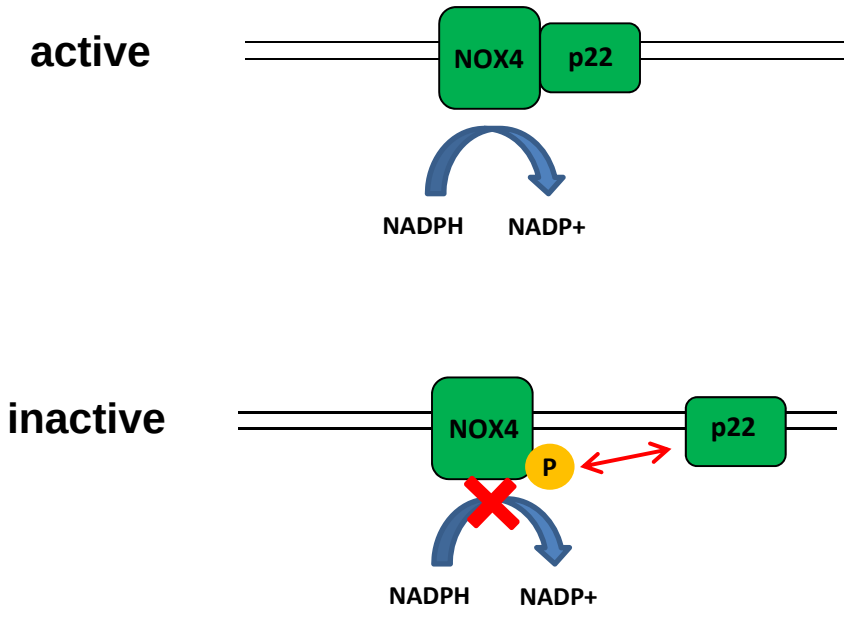
## Supplemental Figure 9

**A**

**N terminal sequence of p22*phox***

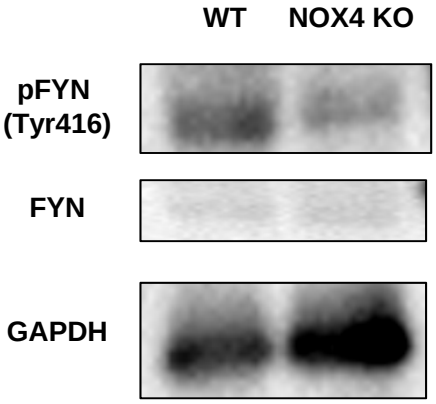
| 1 | | | | | | | | | 10 | | | | | | | | | | 20 | | | | | | | | | | 30 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| m | g | q | l | e | w | a | m | w | a | n | e | q | a | l | a | s | g | l | i | l | i | t | g | g | l | v | a | t | a |

**B**



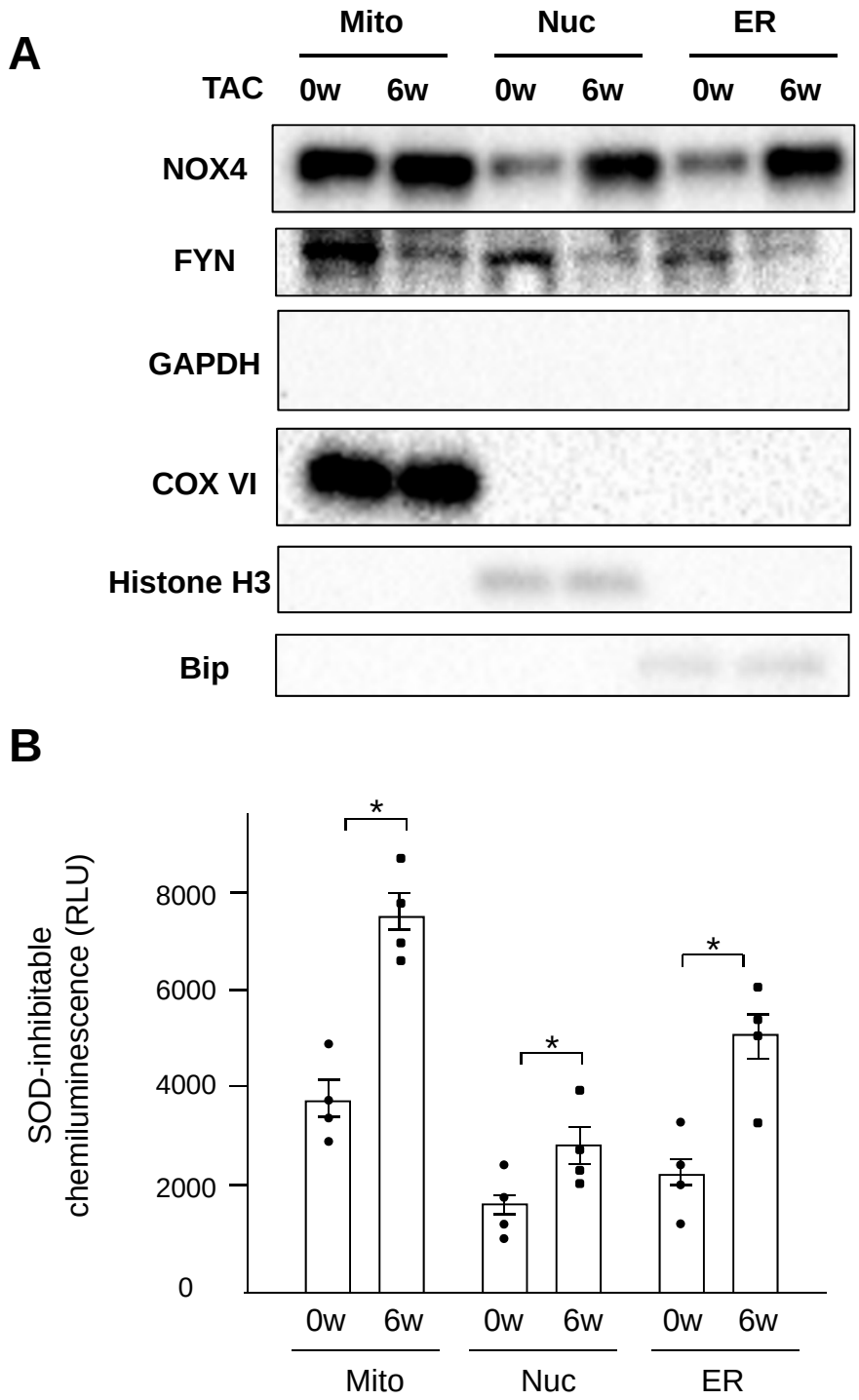

**Supplemental Figure 9 p22*phox* has negatively charged amino acids in its N-terminus. a.** The N terminal amino acid sequence of p22*phox*. **b.** A schematic representation of our hypothesis as to how Y566 phosphorylation of NOX4 inhibits Nox4.

## Supplemental Figure 10



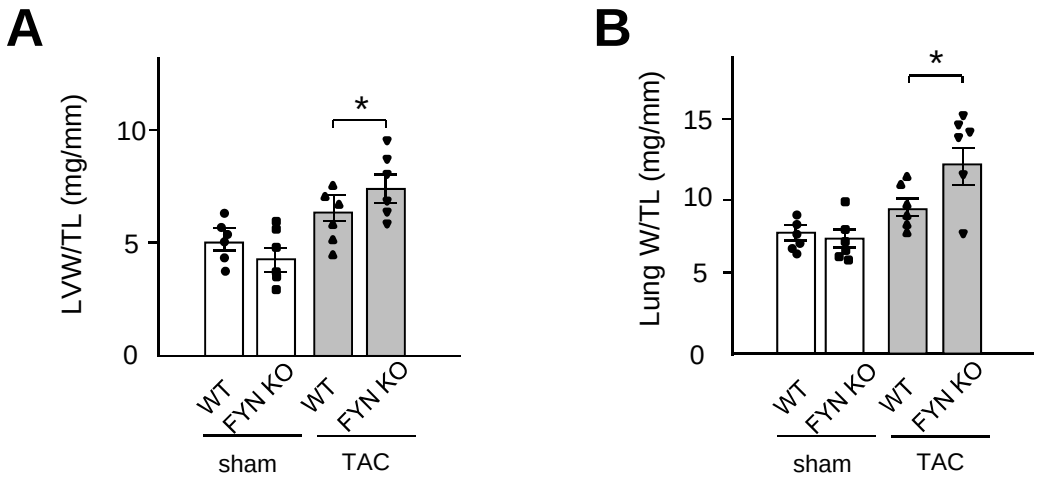

**Supplemental Figure 10**
phosphorylated FYN, total FYN, and GAPDH in LV after in WT and NOX4 KO mice. After immunoprecipitation (IP) with an anti-FYN antibody, immunoblot analyses (IB) for phospho-Src (S416) were performed to detect FYN phosphorylated at the tyrosine in the activation loop of the kinase domain (pFYN). The experiment was conducted 3 times.

## Supplemental Figure 11



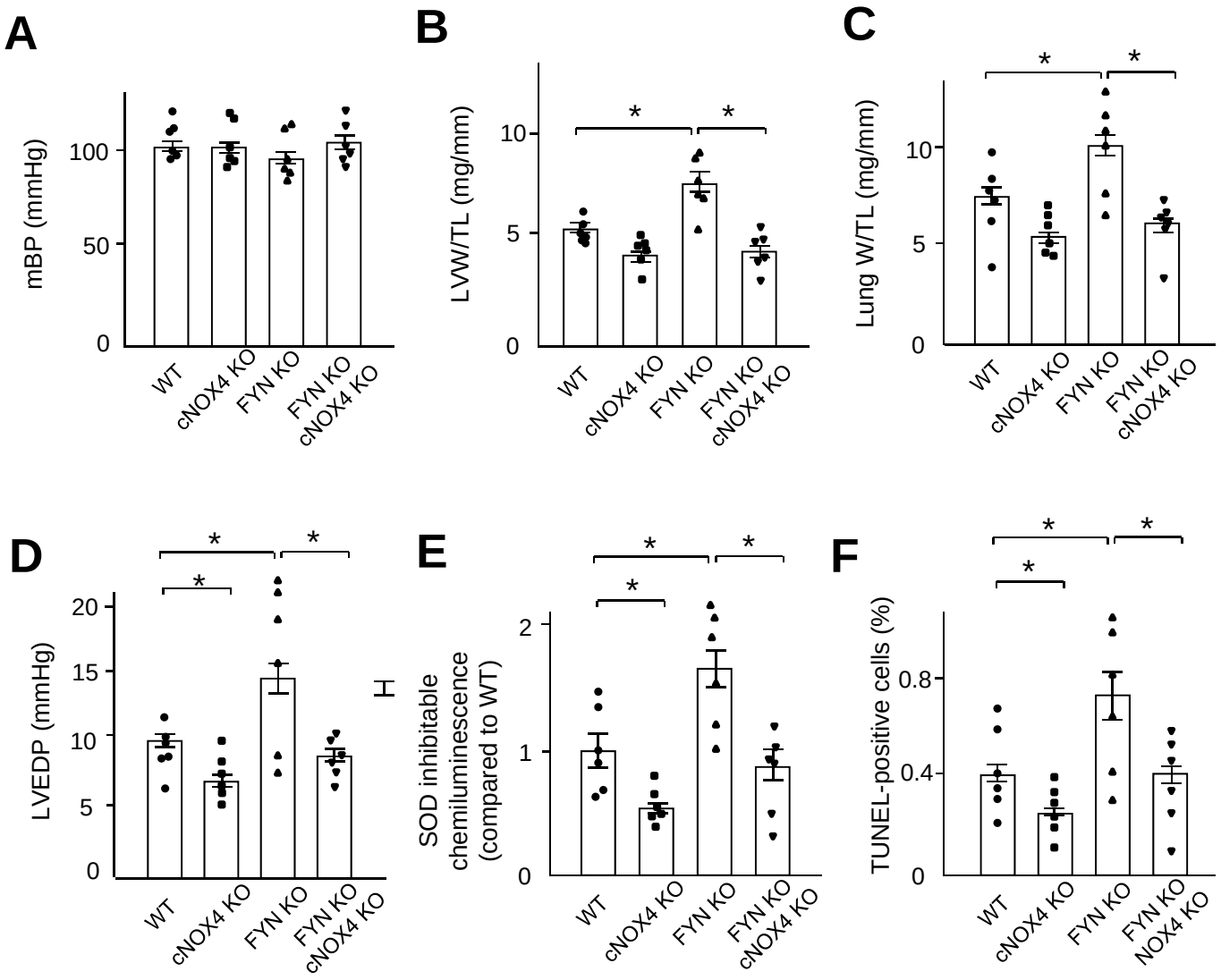

**Supplemental Figure 11 A.** Expression levels of NOX4, FYN, GAPDH, COX IV, Histone H3, and Bip in each fraction from the indicated mouse hearts 0 and 6 weeks after TAC operation. The experiment was conducted 3 times. **B.** NADPH-dependent and SOD-inhibitable $O_2^-$ release in each fraction from the indicated mouse hearts was measured by the lucigenin method (n=4). Statistical analyses were done by one-way ANOVA followed by a post hoc Fisher's comparison test. *****P<0.05.

## Supplemental Figure 12



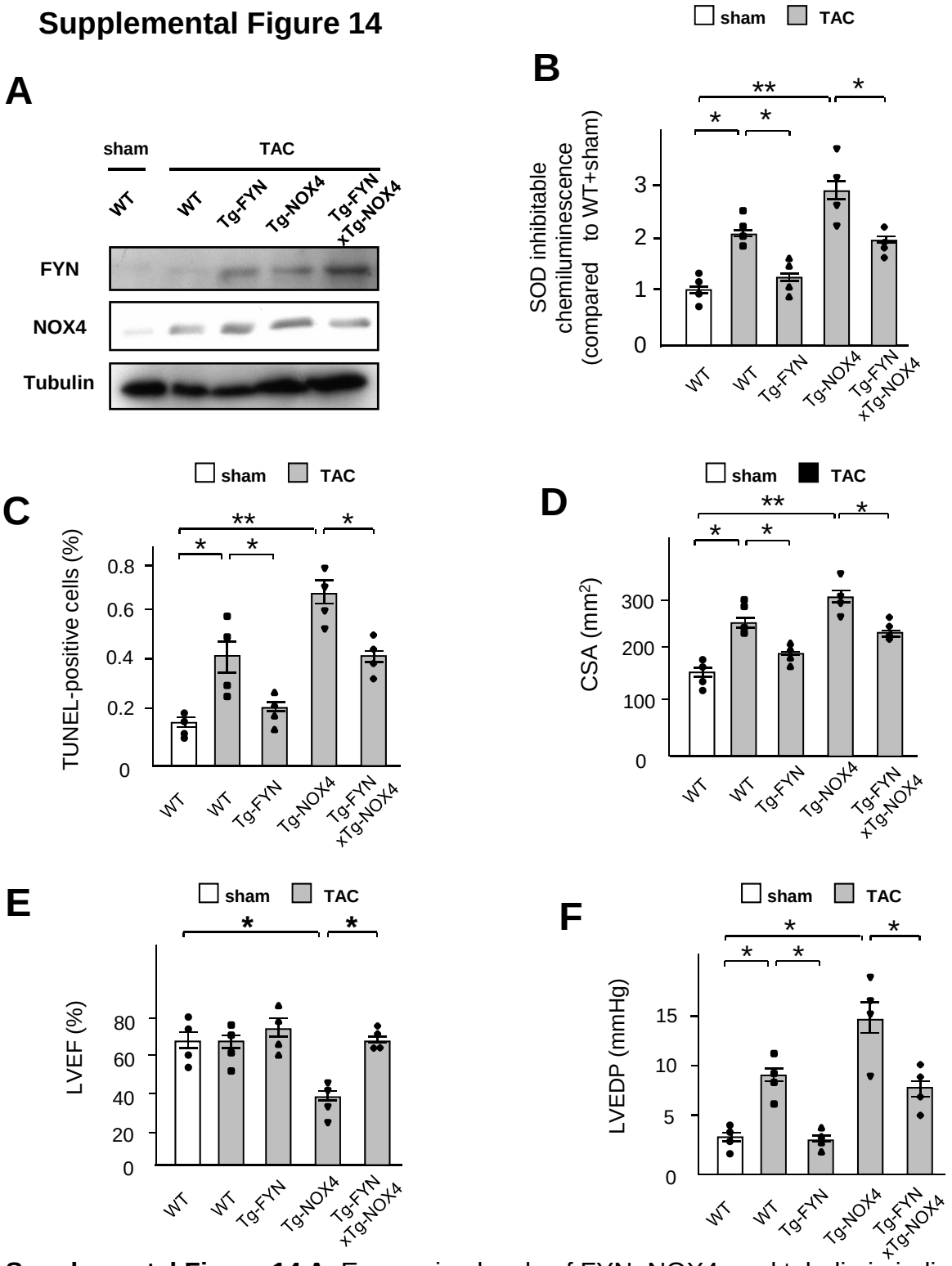

**Supplemental Figure 12. Deletion of FYN promotes LV dysfunction and heart failure in response to pressure overload. A, B.** LVW/TL, and Lung W/TL were determined in the indicated mice 2 weeks after TAC (n=6). Statistical analyses were done by one-way ANOVA followed by a post hoc Fisher's comparison test. *P<0.05

## Supplemental Figure 13



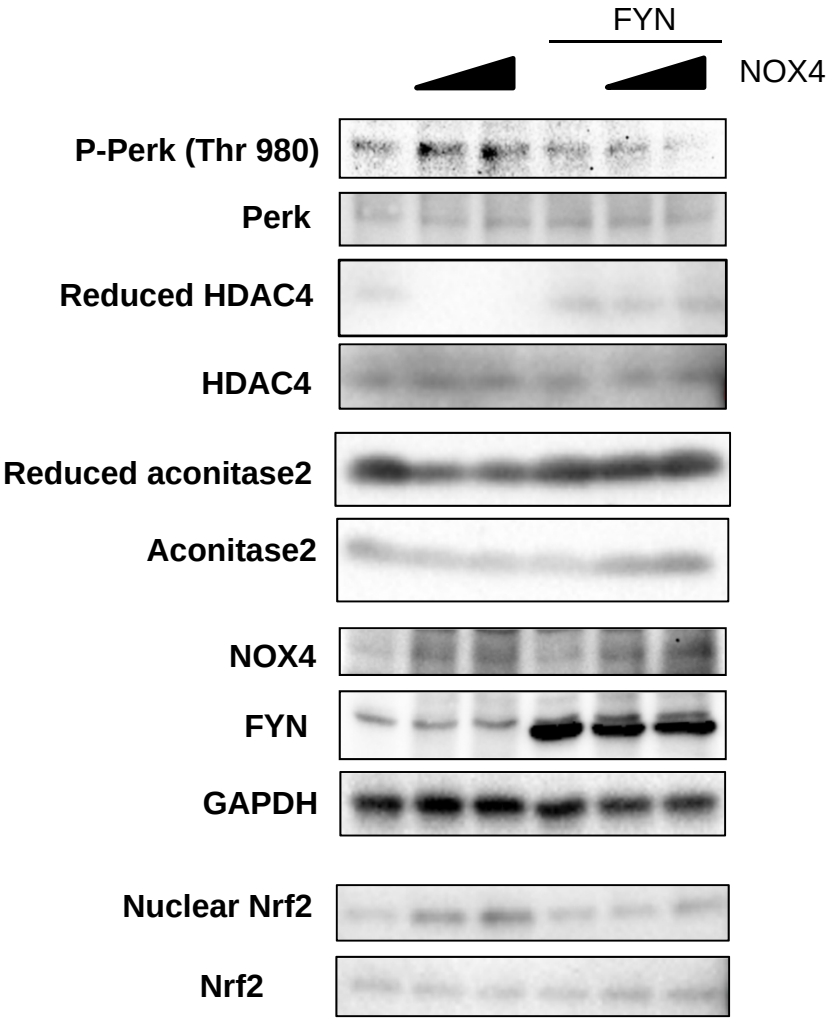

**Supplemental Figure 13. Deletion of NOX4 rescues cardiac dysfunction and heart failure in FYN KO mice in response to PO. A, D.** Mean BP and LV end-diastolic pressure were evaluated with Millar catheter in the indicated mice 2 weeks after TAC operation (n=6). **B, C.** LVW/TL and Lung W/TL were determined in the indicated mice 2 weeks after TAC operation (n=6). **E.** NADPH-dependent and SOD-inhibitable $O_2^-$ release in mitochondrial fraction from the indicated mouse hearts was measured by the lucigenin method (n=6). **F.** Apoptosis in the indicated mouse hearts was evaluated with TUNEL staining (n=6). Statistical analyses were done by one-way ANOVA followed by a post hoc Fisher's comparison test..
*****P<0.05

## Supplemental Figure 14



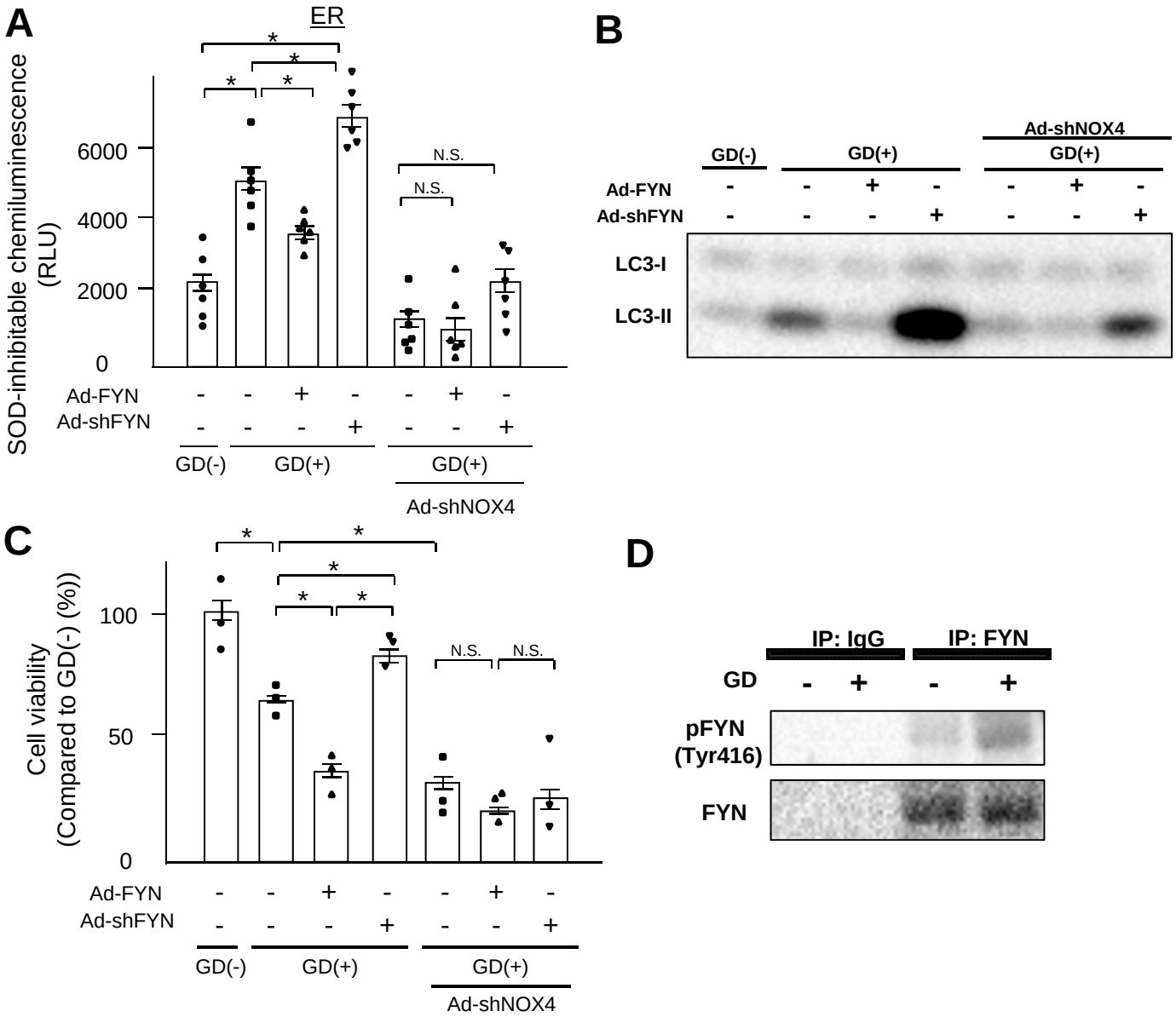

**Supplemental Figure 14 A.** Expression levels of FYN, NOX4, and tubulin in indicated mouse hearts subjected to either sham or TAC operation. The experiment was conducted 3 times. **B.** NADPH-dependent and SOD-inhibitable $O_2^-$ release in mitochondrial fraction from the indicated mouse hearts was measured by the lucigenin method (n=4). **C.** Apoptosis in the indicated mouse hearts was evaluated with TUNEL staining (n=4). **D.** LV CM cross-sectional area (CSA) evaluated with wheat germ agglutinin staining (n=4). **E.** LVEF was evaluated with echocardiography (n=4). **F.** LVEDP was evaluated with a Millar catheter (n=4). Statistical analyses were done by one-way ANOVA followed by a post hoc Fisher's comparison test. *****P<0.05, ******P<0.01.

## Supplemental Figure 15



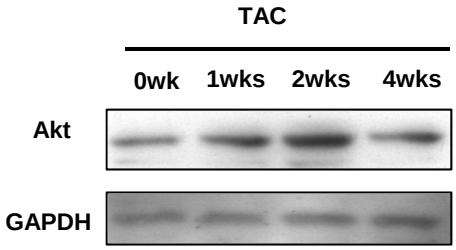

**Supplemental Figure 15** Protein levels of phosphorylated Perk at Thr980, Perk, reduced HDAC4, HDAC4, reduced aconitase2, aconitase2, NOX4, FYN, GAPDH, nuclear Nrf2, and Nrf2 in CMs transduced with indicated adenoviruses was determined. The extent of cysteine reduction in HDAC4 and aconitase2 in CMs transduced with indicated adenoviruses was detected by iodoacetamide assay. The experiment was conducted 3 times.

## Supplemental Figure 16




**Supplemental Figure 16 A.** The cells were pre-incubated with the indicated adenovirus for 48 hours and then the cells we cultured with normal or glucose-free medium for 4 hours. NADPH-dependent $O_2^-$ release was measured by the lucigenin method. The SOD-inhibitable component of $O_2^-$ release from the ER fraction in CMs transduced with indicated adenoviruses was determined (n=6). **B.** The cells were pre-incubated with the indicated adenovirus for 48 hours and then the cells were cultured with normal or glucose-free medium for 4 hours. Protein levels of LC3-I and LC3-II in cultured neonatal rat CMs transduced with indicated adenoviruses (n=3). The experiment was conducted 3 times. **C.** The cells were pre-incubated with the indicated adenovirus for 48 hours and then the cells we cultured with normal or glucose-free medium for 24 hours. Viable cell numbers were measured by CTB assays according to the supplier's protocol (n=8). **D**. The cells were cultured with normal or glucose-free medium for 4 hours. The expression levels of phosphorylated FYN and total FYN were evaluated with immunoblotting. After immunoprecipitation (IP) with an anti-Fyn antibody, immunoblot analyses (IB) for phospho-Src (S416) were performed to detect FYN phosphorylated at the tyrosine in the activation loop of the kinase domain (pFYN). GD, glucose deprivation. The experiment was conducted 3 times. Statistical analyses were done by one-way ANOVA followed by a post hoc Fisher's comparison test. *P<0.05.

## Supplemental Figure 17




**Supplemental Figure 17** Protein levels of Akt and GAPDH in LV after TAC at various time points. The experiment was conducted 3 times.

**Supplemental Table 1**

## Echocardiographic data of WT and FYN KO mice 2 weeks after TAC operation

| | WT+Sham n=6 | FYN KO+Sham n=6 | WT+TAC n=8 | FYN KO+TAC n=8 |
|---|---|---|---|---|
| **HR, bpm** | 511±11 | 517±15 | 500±15 | 493±10 |
| **LVEDD, mm** | 3.5±0.1 | 3.5±0.1 | 3.2±0.09*[1] | 4.0±0.09*[2] |
| **LVESD, mm** | 2.2±0.1 | 2.1±0.2 | 1.8±0.2*[1] | 3.3±0.1*[2] |
| **LVEF, %** | 74.7±5.3 | 76.7±5.6 | 79.5±5.2 | 44.3±4.6*[2] |
| **IVSWT, mm** | 0.73±0.02 | 0.74±0.03 | 1.08±0.04*[1] | 1.25±0.05*[2] |
| **PWT, mm** | 0.72±0.03 | 0.73±0.03 | 1.06±0.06*[1] | 1.20±0.07*[2] |

HR, heart rate; LV, left ventricle; EDD, end-diastolic dimension; ESD, end-systolic dimension; EF, ejection fraction; EDP, end-diastolic pressure; IVSWT, interventricular septal wall thickness; PWT, posterior wall thickness.
Data are mean ± SEM, *[1] $P<0.05$ vs WT+Sham, *[2] $P<0.05$ vs WT+TAC.

**Supplemental Table 2**

## Hemodynamic analyses of WT and FYN KO mice 2 weeks after TAC operation

| | WT+Sham n=6 | FYN KO+Sham n=6 | WT+TAC n=8 | FYN KO+TAC n=8 |
|---|---|---|---|---|
| **HR, bpm** | 497±18 | 503±19 | 494±17 | 481±18 |
| **sBP, mmHg** | 99±4 | 100±4 | 161±6*[1] | 156±6*[1] |
| **dBP, mmHg** | 67±3 | 64±4 | 71±5 | 70±4 |
| **mBP, mmHg** | 77±2 | 76±3 | 101±4*[1] | 99±4*[1] |
| **Peak LVP, mmHg** | 102±3 | 99±4 | 157±8*[1] | 160±5*[1] |
| **LVEDP, mmHg** | 2.6±0.7 | 3.0±0.7 | 9.9±1.0*[1] | 15.3±1.9*[2] |
| **+dP/dt, mmHg/s** | 6733±402 | 7033±578 | 7663±566 | 4825±432*[2] |
| **-dP/dt , mmHg/s** | 6517±423 | 6467±786 | 4875±275*[1] | 3625±339*[2] |

HR, heart rate; sBP, systolic blood pressure; dBP, diastolic blood pressure; mBP, mean blood pressure; LV, left ventricle; EDP, end-diastolic pressure. Data are mean ± SEM, *[1] $P<0.05$ vs WT+Sham, *[2] $P<0.05$ vs WT+TAC.

**Supplemental Table 3**

## Echocardiographic data of WT, cNOX4 KO, FYN KO, and double KO mice 2 weeks after TAC operation

| | WT+TAC n=6 | cNOX4 KO+TAC n=6 | FYN KO+TAC n=6 | DKO+TAC n=6 |
|---|---|---|---|---|
| **HR, bpm** | 501±16 | 489±15 | 531±20 | 496±14 |
| **LVEDD, mm** | 3.3±0.06 | 3.2±0.2 | 3.8±0.1*[1] | 3.4±0.1*[2] |
| **LVESD, mm** | 2.0±0.1 | 2.0±0.2 | 3.1±0.1*[1] | 2.3±0.1*[2] |
| **LVEF, %** | 75.5±4.0 | 76.7±3.1 | 44.6±3.6*[1] | 68.0±4.0*[2] |
| **IVSWT, mm** | 1.06±0.04 | 0.9±0.04*[1] | 1.20±0.06*[1] | 1.06±0.06*[2] |
| **PWT, mm** | 0.98±0.12 | 0.77±0.03*[1] | 1.18±0.08 *[1] | 1.02±0.06*[2] |

DKO, double knock-out (FYNKO-cNOX4KO); HR, heart rate; LV, left ventricle; EDD, end-diastolic dimension; ESD, end-systolic dimension; EF, ejection fraction; EDP, end-diastolic pressure; IVSWT, interventricular septal wall thickness; PWT, posterior wall thickness. Data are mean ± SEM, *[1] P<0.05 vs WT+TAC, *[2] P<0.05 vs FYN KO+TAC.

**Supplemental Table 4**

**Hemodynamic analyses of WT, cNOX4 KO, FYN KO, and double KO mice 2 weeks after TAC operation**

| | WT+TAC n=6 | cNOX4 KO+TAC n=6 | FYN KO+TAC n=6 | DKO+TAC n=6 |
|---|---|---|---|---|
| **HR, bpm** | 489±26 | 473±25 | 463±29 | 504±18 |
| **sBP , mmHg** | 158±5 | 163±7 | 157±5 | 166±6 |
| **dBP, mmHg** | 72±5 | 70±3 | 67±4 | 75±6 |
| **mBP, mmHg** | 100±2 | 101±3 | 98±3 | 105±5 |
| **Peak LVP, mmHg** | 163±6 | 157±8 | 162±10 | 160±8 |
| **LVEDP, mmHg** | 9.5±0.9 | 7.1±0.7*[1] | 16.2±2.6*[1] | 8.7±0.8*[2] |
| **+dP/dt, mmHg/s** | 7717±620 | 8133±392 | 4650±367*[1] | 6717±410*[2] |
| **-dP/dt, mmHg/s** | 4683±304 | 5883±295*[1] | 3383±386*[1] | 4717±289*[2] |

DKO, double knockout; HR, heart rate; sBP, systolic blood pressure; dBP, diastolic blood pressure; mBP, mean blood pressure; LV, left ventricle; EDP, end-diastolic pressure.

Data are mean ± SEM, *[1] $P<0.05$ vs WT+TAC, *[2] $P<0.05$ vs FYN KO+TAC.

## Supplemental Table 5

### Echocardiographic data of WT, Tg-NOX4, Tg-FYN and bigenic mice 2 weeks after TAC operation

| | WT+Sham n=4 | WT+TAC n=4 | Tg-FYN+TAC n=4 | Tg-NOX4+TAC n=4 | Bigenic+TAC n=4 |
|---|---|---|---|---|---|
| **HR, bpm** | 497±25 | 496±23 | 551±31 | 522±15 | 506±14 |
| **LVEDD, mm** | 3.4±0.09 | 3.1±0.1 | 3.4±0.1 | 3.9±0.1*[2] | 3.5±0.09*[3] |
| **LVESD, mm** | 2.3±0.2 | 2.1±0.2 | 2.1±0.2 | 3.2±0.2*[2] | 2.3±0.1*[3] |
| **LVEF, %** | 68.3±6.3 | 66.8±5.0 | 73.5±6.1 | 43.5±4.6*[2] | 70.8±3.7*[3] |
| **IVSWT, mm** | 0.74±0.04 | 1.15±0.06*[1] | 0.88±0.08*[2] | 1.30±0.11*[2] | 1.12±0.07*[3] |
| **PWT, mm** | 0.73±0.03 | 1.20±0.04*[1] | 0.89±0.05*[2] | 1.35±0.06*[2] | 1.10±0.07*[3] |

HR, heart rate; LV, left ventricle; EDD, end-diastolic dimension; ESD, end-systolic dimension; EF, ejection fraction; EDP, end-diastolic pressure; IVSWT, interventricular septal wall thickness; PWT, posterior wall thickness. Data are mean ± SEM, *[1] $P<0.05$ vs WT+Sham, *[2] $P<0.05$ vs WT+TAC, *[3] $P<0.05$ vs Tg-NOX4+TAC.

## Supplemental Table 6

### Hemodynamic analyses of WT, Tg-NOX4, Tg-FYN and bigenic mice 2 weeks after TAC operation

| | WT+Sham n=4 | WT+TAC n=4 | Tg-FYN+TAC n=4 | Tg-NOX4+TAC n=4 | Bigenic+TAC n=4 |
|---|---|---|---|---|---|
| **HR, bpm** | 478±10 | 505±13 | 493±32 | 459±37 | 495±26 |
| **sBP, mmHg** | 97±7 | 154±5*[1] | 151±11*[1] | 146±9*[1] | 150±6*[1] |
| **dBP, mmHg** | 68±5 | 76±4 | 75±3 | 75±3 | 72±7 |
| **mBP, mmHg** | 79±3 | 102±4*[1] | 101±5*[1] | 99±5*[1] | 98±4*[1] |
| **Peak LVP, mmHg** | 99±3 | 159±9*[1] | 154±9*[1] | 151±11*[1] | 164±5*[1] |
| **LVEDP, mmHg** | 3.3±0.3 | 9.2±1.3*[1] | 3.3±0.3*[2] | 14.0±2.4*[2] | 8.3±0.9*[3] |
| **+dP/dt, mmHg/s** | 8425±649 | 6925±660 | 7525±390 | 3513±401*[2] | 6550±585*[3] |
| **-dP/dt, mmHg/s** | 6425±622 | 4350±340*[1] | 6025±487*[2] | 3263±626*[2] | 5325±309*[3] |

HR, heart rate; sBP, systolic blood pressure; dBP, diastolic blood pressure; mBP, mean blood pressure; LV, left ventricle; EDP, end-diastolic pressure. Data are mean ± SEM, *[1] $P<0.05$ vs WT+Sham, *[2] $P<0.05$ vs WT+TAC, *[3] $P<0.05$ vs Tg-NOX4+TAC.