Online Supplement

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Control of p21^{Cip} by BRAP is Critical for Cardiomyocyte Cell Cycle Progression and Survival

Supplementary methods

Generation of animal models. To generate conditional *Brap* knockout mice, a targeting vector was designed to display a long homologous region of 5.9 kb and a short homologous region of 2.6 kb, isogenic with the *Brap* locus in the 129Sv/Pas ES cell line that was used for recombination. Two loxP (fl) sites were inserted, flanking exon 6 and leading to a frame shift and the generation of a premature stop codon in exon 7 after Cre recombination. The linearized targeting vector was transfected into ES cells according to standard electroporation procedures. A neomycin selection cassette flanked by frt sites and a diphteria toxin cassette, which was used as a negative selection marker, were deleted after successful homologous recombination. To delete *Brap* selectively in cardiomyocytes, Brap$^{fl/fl}$ mice were interbred with αMHC-Cre mice[1]. As control groups we analysed: αMHC-Cre^{-}/Brap$^{wt/wt}$, αMHC-Cre^{+}/Brap$^{wt/wt}$and αMHC-Cre^{-}/Brap$^{fl/fl}$ littermates to exclude possible side-effects secondary to Cre expression or to loxP insertion. To delete BRAP selectively in cardiomyocytes during early cardiac development, Brap$^{fl/fl}$ mice were interbred with Nkx2-5-Cre TG mice [2] and with Mlc2V-Cre mice. For inducible BRAP deletion in adult cardiomyocytes we interbred Brap$^{fl/fl}$ mice with αMHC-MerCreMer mice (B6.FVB(129)-*A1cf*$^{Tg(Myh6\text{-}cre/Esr1*)1Jmk}$/J) and induced Cre expression in 10 week old mice by injection of 40 mg/kg BW tamoxifen on 5 days. Analysis was performed 3 weeks after the end of injection. In addition to the conditional knockout mice, we also generated non-tissue specific (general) *Brap*-deleted mice from mouse embryonic stem cells carrying a gene trap in the *Brap* locus

(SIGTR cell line ID CD0202) using blastocyst injection according to a standard protocol (The Wellcome Trust Sanger Institute, U.K.). Finally, for enhanced expression of BRAP, transgenic mice were generated by ligation of a cDNA, encoding the 592-amino acid BRAP and a Flag tag, into the minimalized αMHC promoter clone 26 (which was kindly provided by J. Robbins), which was earlier demonstrated to evoke cardiomyocyte specific expression [1]. Following the excision of prokaryotic DNA, the construct was microinjected into the pronucleus of FVB/N mouse oocytes and implanted into pseudo-pregnant females according to standard techniques.

Histology and cell cycle analysis. Adult hearts were isolated following sacrificing the mice by anesthetic overdose with isoflurane (Abbott) and subsequent cervical dislocation. Neonatal mice were sacrificed by decapitation after deep anesthesia with isoflurane. For a histological examination of the mouse embryos, pregnant mice were sacrificed by cervical dislocation on ED 8.5. The embryos were removed from the uterus, transferred into a Petri dish with Locke solution and dissected under a dissection microscope. The extra-embryonic membranes were removed and used for genotyping. Embryos were rinsed in calcium-free Locke solution of manganese chloride (20 mM) to induce cardiac arrest in a dilated state. After cardiac arrest, embryos were fixed in Bouin's solution. The specimens were dehydrated in a graded ethanol series, cleared in methyl benzoate and embedded in Parablast (Sherwood). Serial transverse sections were cut at 7 µm, transferred to albumin/glycerin coated glass slides and stained with hematoxylin and eosin according to Harris. For the analysis of DNA synthesis *in vivo*, 4-day-old mice were given an intraperitoneal 5'-Bromo-2'-deoxyuridine (BrdU) (Sigma-Aldrich) injection (120 mg/kg body weight). Three hours after injection, the mice were sacrificed, and the hearts were fixed with 4% paraformaldehyde. Paraffin sections were stained with antibodies against BrdU (Abcam), Troponin T (ThermoFisher), phospho-HistonH3 (Millipore), $p21^{Cip}$ (Abcam), Ki-67 (Invitrogen), PCM-1 (Sigma-Aldrich) and

Alexa Fluor-488 and -555 labeled secondary antibodies (ThermoFisher). Nuclei were visualized by DAPI staining.

For quantification of mono- and bi-nucleated cardiomyocytes we isolated neonatal cardiomyocytes from 4 day old mice. Hearts were dissected and subsequently digested in 1 ml 0.1% Collagenase II (Worthington) (in sterile PBS) per heart for 18 min at 37°C (shaking). Afterwards, 1ml medium (DMEM incl. Glucose 4,5 g/l + 15% FCS + 1% P/S + 1% non-essential amino acids) was added and cells were washed two times with medium. Cells were plated and cultivated overnight on gelatin-coated chamber slides. Cells were washed with PBS and fixation was performed with Methanol/Aceton (10 min, -20°C). For identification of cardiomyocytes, cells were stained with sarcomeric α-actinin antibody (Sigma-Aldrich) and nuclei were visualized with DAPI.

Forcell cycle analysis by flow cytometry, cardiomyocytes were enzymatically isolated using collagenase type II (Worthington) as specified above and stained with sarcomeric α-actinin antibody (Sigma-Aldrich) and FITC-labeled secondary antibody to discriminate cardiomyocytes from other cell types, followed by propidium iodide staining (Sigma-Aldrich). The cells were analyzed using a FACS Canto II flow cytometer and FACS Diva software (BD Biosciences, Germany). All analyses were conducted with the examiner blinded towards the group assignment.

Western Blot and Real-time RT PCR. Protein lysates from homogenized mouse hearts were subjected to Western Blot analysis using antibodies against GAPDH (Bio Trend) , BRAP2 (custom made, Synaptic Systems), B-Raf (Cell Signaling), phospho-(Ser455)-B-Raf (Cell Signaling), C-Raf (santa cruz), phospho-(Ser338)-C-Raf (Cell Signaling). For Real-Time RT PCR, RNA was isolated from left ventricular heart tissue using the RNeasy Fibrous Tissue Mini Kit (Qiagen). Complementary DNA (cDNA) was synthesized from 200 ng of total RNA, using the iScript cDNA Synthesis Kit (Bio-Rad), and Real-

Time RT PCR was performed using IQ SYBR Green Supermix and the iCycler iQ5 Detection System (Bio-Rad). Data were normalized against the mean of Hsp90ab1 and GAPDH or 18S RNA, and quantitation was conducted using the delta-delta CT method.

Supplemental References

1. Agah R, Frenkel PA, French BA, Michael LH, Overbeek PA, Schneider MD. Gene recombination in postmitotic cells. Targeted expression of Cre recombinase provokes cardiac-restricted, site-specific rearrangement in adult ventricular muscle in vivo. *J Clin Invest* 1997;**100**:169-179.

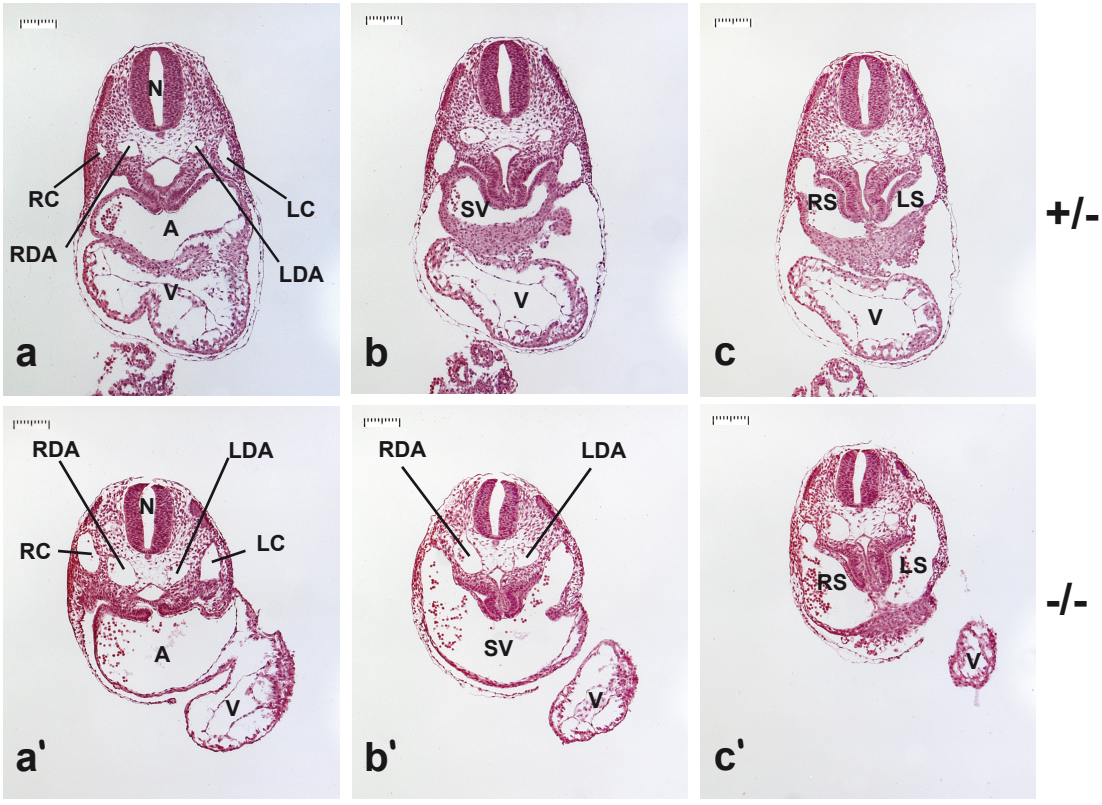
2. Moses KA, DeMayo F, Braun RM, Reecy JL, Schwartz RJ. Embryonic expression of an Nkx2-5/Cre gene using ROSA26 reporter mice. *Genesis* 2001;**31**:176-180.

Supplemental Fig. 1

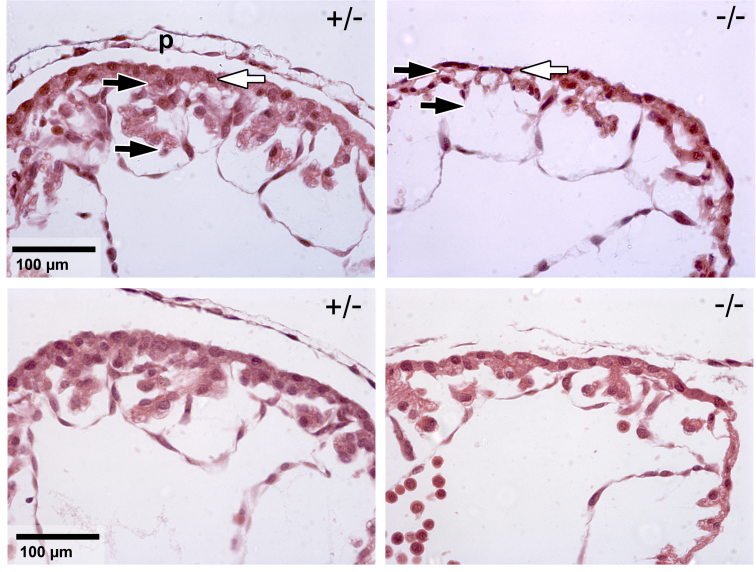
A general *Brap* KO

	P0	%
***Brap* +/+**	32	32.0
***Brap* +/ -**	68	68.0
***Brap* -/-**	0	0.0
total	100	100.0

B



C



Supplemental Fig. 2

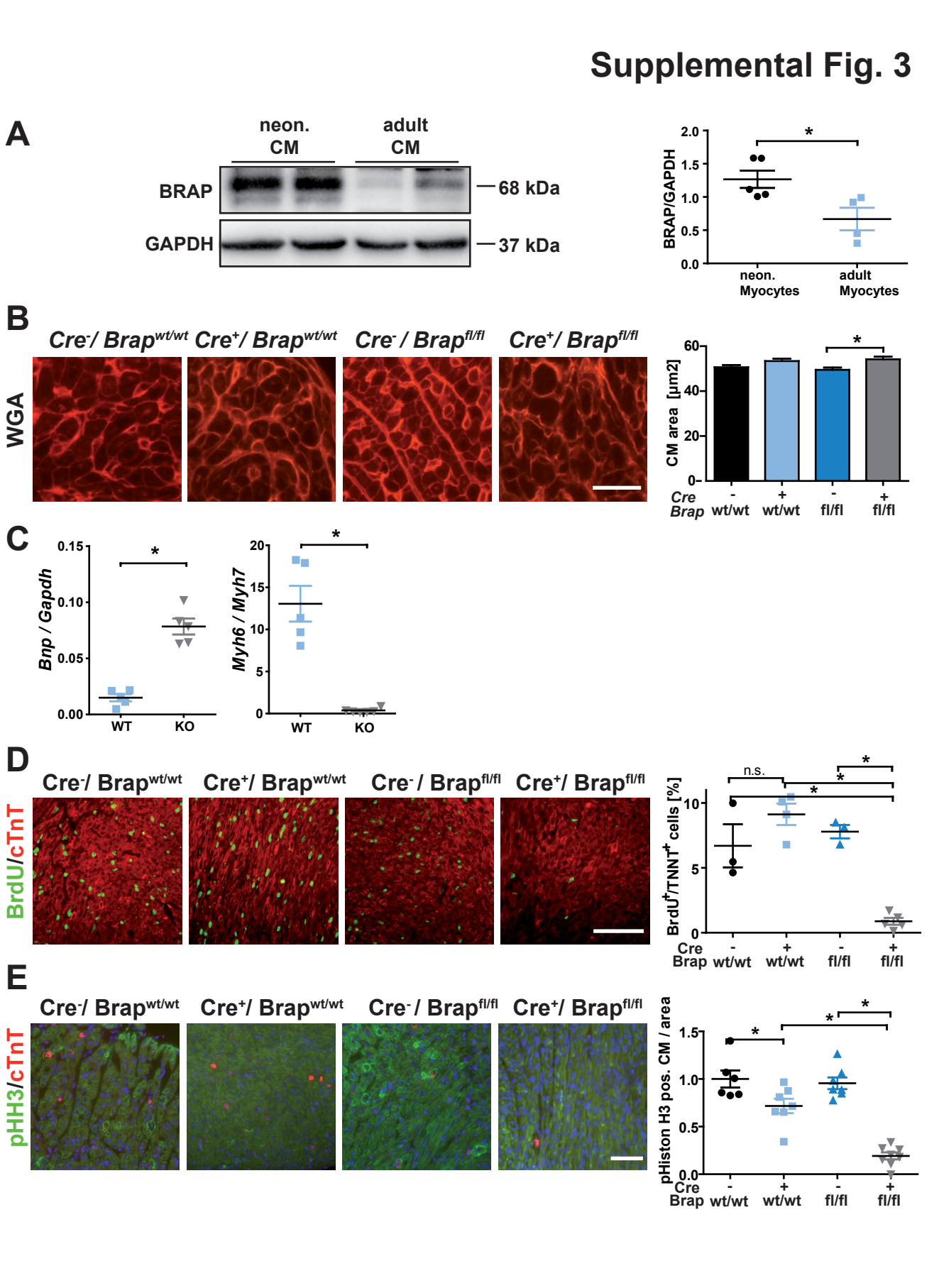
A

Genotype Nkx2.5-Cre	mice (4w)	%
***Cre*- / *Brap* wt/wt**	6	8.5
***Cre*+ / *Brap* wt/wt**	13	18.3
***Cre*- / *Brap* fl/wt**	10	14.1
***Cre*+ / *Brap* fl/wt**	10	14.1
***Cre*- / *Brap* fl/fl**	9	12.7
***Cre*+ / *Brap* fl/fl**	0	0.0
total	48	100.0

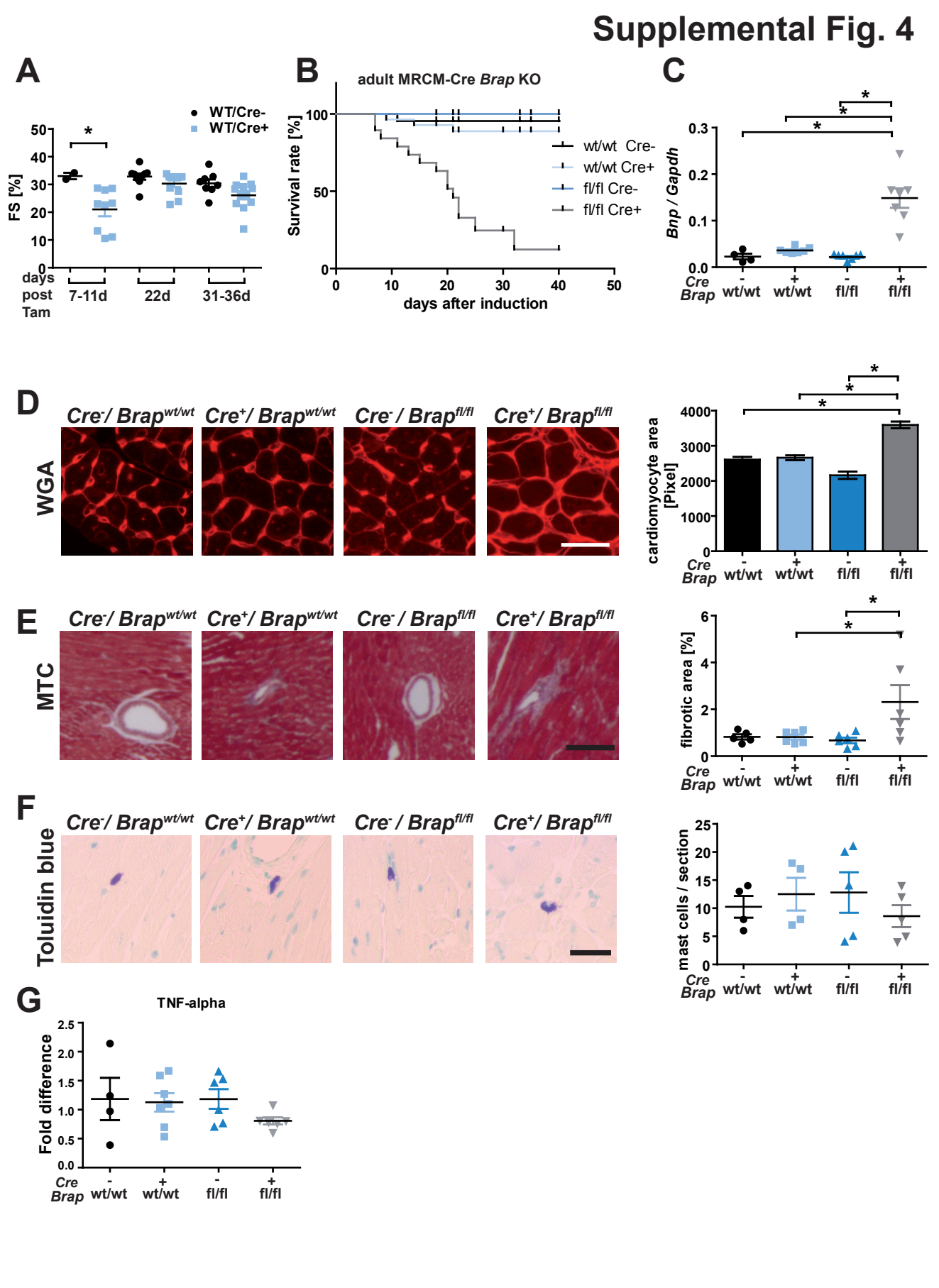
B

Genotype Mlc2V-Cre	mice (4w)	%
***Cre*- / *Brap* wt/wt**	2	4.4
***Cre*+ / *Brap* wt/wt**	12	26.7
***Cre*- / *Brap* fl/wt**	11	24.4
***Cre*+ / *Brap* fl/wt**	7	15.6
***Cre*- / *Brap* fl/fl**	13	28.9
***Cre*+ / *Brap* fl/fl**	0	0.0
total	45	100.0

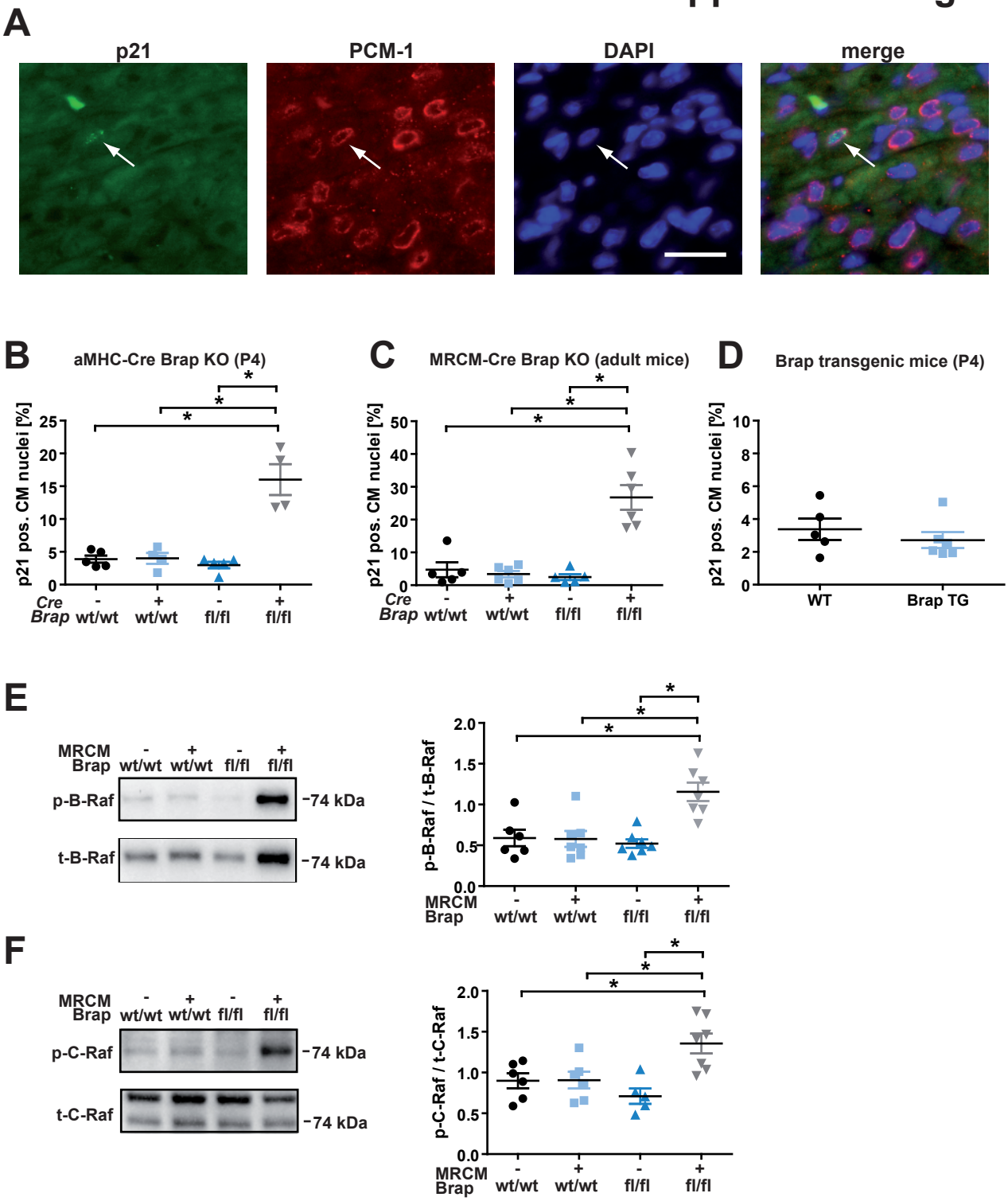
Supplemental Fig. 3



Supplemental Fig. 4



Supplemental Fig. 5



Supplemental Fig. 6