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Supplementary Materials for

Autophagy induction in atrophic muscle cells requires ULK1 activation by TRIM32 through unanchored K63-linked polyubiquitin chains

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This PDF file includes:

SUPPLEMENTARY FIGURES

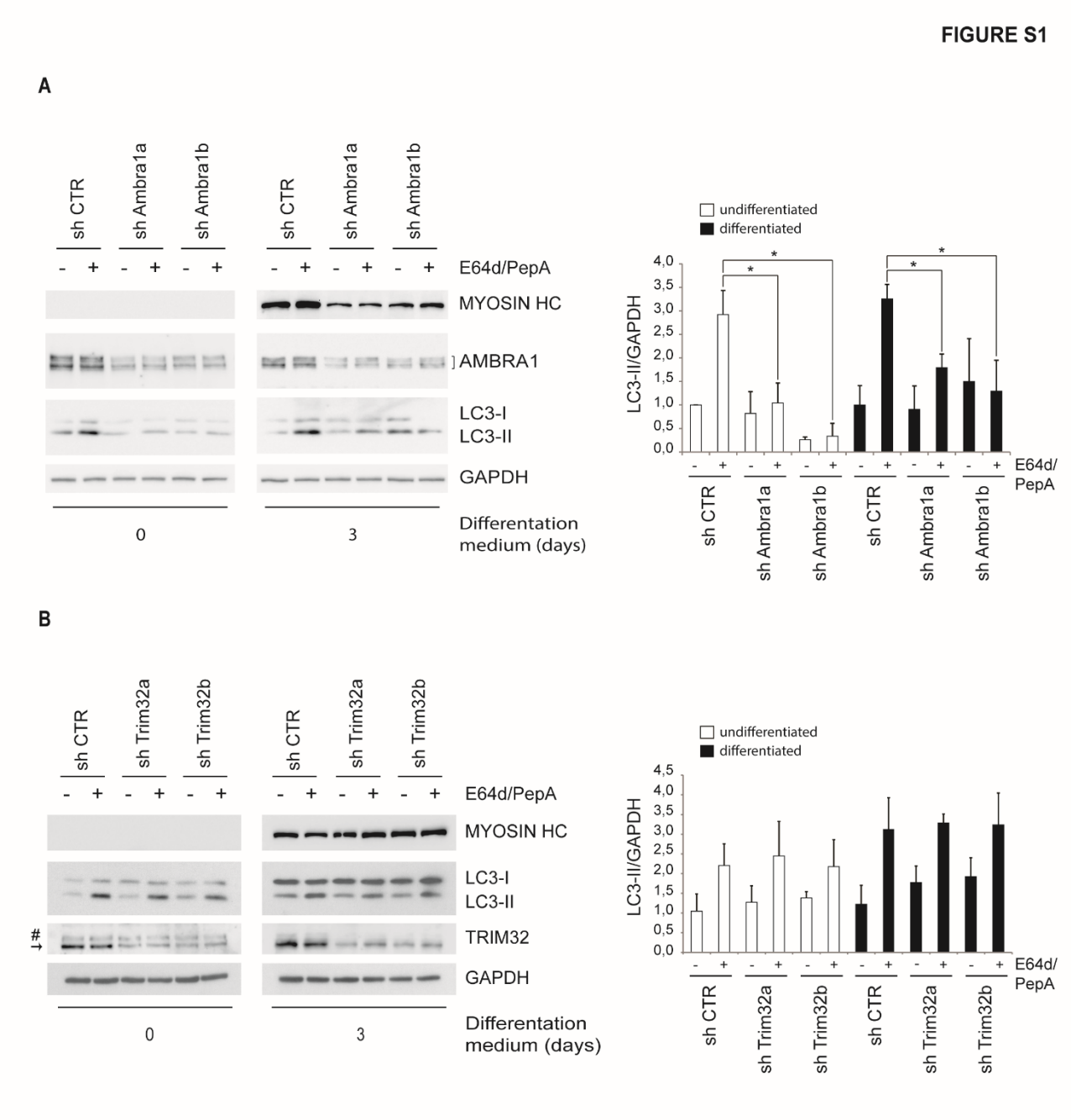
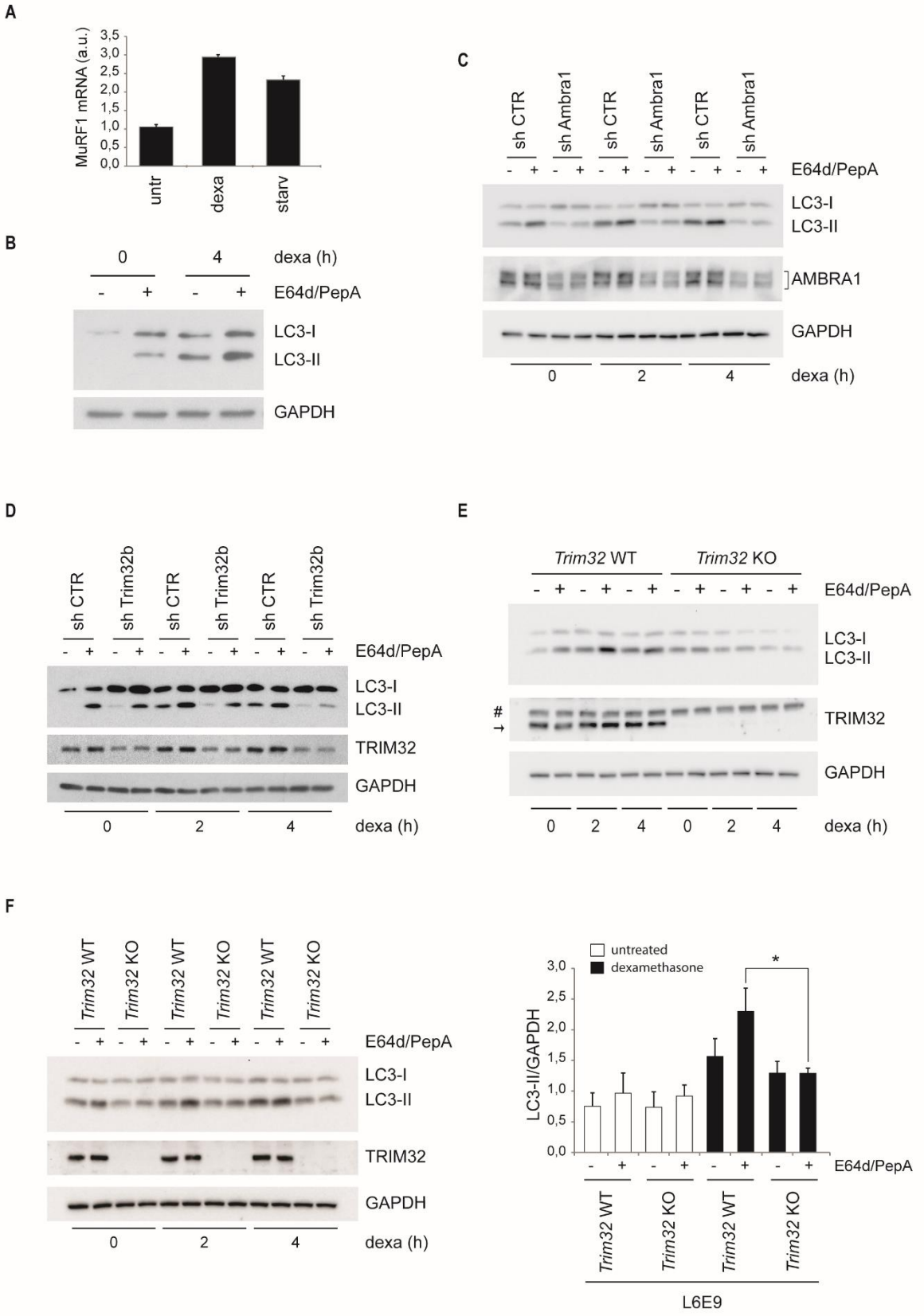


Fig. S1. AMBRA1, but not TRIM32, is required for basal autophagy in myoblast cells. **A)** C2.7 cells infected with shCTR, shAmbra1a, shAmbra1b were cultured either in proliferative conditions (20% FCS) or in differentiation medium (2% HS) for 3 days, and incubated with the lysosome inhibitors E64d and pepstatin A (E64d/PepA) for 1 hour before lysis to monitor autophagy flux, as indicated. LC3, AMBRA1 and MYOSIN HEAVY CHAIN (MYOSIN HC) levels were analyzed by immunoblotting using specific antibodies (left

panel). GAPDH was included as a loading control. Graph (right panel) reports means ± s.d. of LC3-II/GAPDH values from 3 independent experiments; * = $p<0.05$. **B)** C2.7 cells infected with shCTR, shTrim32a and shTrim32b were analysed as described in (A). LC3, Trim32 and Myosin HC levels were analyzed by immunoblotting using specific antibodies (left panel). Arrow points to TRIM32 specific band; # indicates a non-specific signal. Graph (right panel) reports means ± s.d. of LC3-II/GAPDH values from 3 independent experiments.

FIGURE S2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MuRF1 mRNA (a.u.)
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2,5
2,0
1,5
1,0
0,5
0,0
untr
dexa
starv
B
0
4
dexa (h)
- + - +
E64d/PepA
LC3-I
LC3-II
GAPDH
C
sh CTR
sh Ambra1
sh CTR
sh Ambra1
sh CTR
sh Ambra1
- + - + - + - + - + - +
E64d/PepA
LC3-I
LC3-II
AMBRA1
GAPDH
0
2
4
dexa (h)
D
sh CTR
sh Trim32b
sh CTR
sh Trim32b
sh CTR
sh Trim32b
- + - + - + - + - + - +
E64d/PepA
LC3-I
LC3-II
TRIM32
GAPDH
0
2
4
dexa (h)
E
Trim32 WT
Trim32 KO
- + - + - + - + - + - +
E64d/PepA
LC3-I
LC3-II
#
TRIM32
GAPDH
0
2
4
0
2
4
dexa (h)
F
Trim32 WT
Trim32 KO
Trim32 WT
Trim32 KO
Trim32 WT
Trim32 KO
- + - + - + - + - + - +
E64d/PepA
LC3-I
LC3-II
TRIM32
GAPDH
0
2
4
dexa (h)
untreated
dexamethasone
*
LC3-II/GAPDH
3,0
2,5
2,0
1,5
1,0
0,5
0,0
- + - + - + - +
E64d/PepA
Trim32 WT
Trim32 KO
Trim32 WT
Trim32 KO
L6E9

Fig. S2. Role of TRIM32 and AMBRA1 in autophagy induction by atrophic stimuli in muscle cells. A) Real-Time PCR analysis of MuRF1 mRNA levels in C2.7 cells treated with dexamethasone or nutrient starved for 24 hours. A.U.= Arbitrary Unit. Expression levels were normalized on the basis of GAPDH values. **B)** C2.7 cells were treated with dexamethasone for 4 hours (h) and incubated, or not, with the lysosome inhibitors E64d/Pepstain A 1 hour before lysis. LC3 protein levels were analysed by immunoblotting. GAPDH was included as a loading control. **C)** shCTR and shAmbra1 cells, cultured in differentiation medium for 3 days, were treated, or not, with dexamethasone for 2 or 4 hours (h). One hour before lysis, cells were incubated with the lysosome inhibitors E64d and Pepstatin A (E64d/PepA) to monitor autophagy flux, as indicated. LC3-II and TRIM32 levels were analysed by immunoblotting. GAPDH was included as a loading control. **D)** shCTR and shTrim32b cells, cultured in differentiation medium for 3 days, were treated, or not, with dexamethasone for 2 or 4 hours (h). Autophagy flux was analysed as described in (C). **E)** *Trim32* KO C2.7 cells were obtained by CRISPR-CAS9 as described in Methods. Cells, cultured in differentiation medium for 3 days, were treated, or not, with dexamethasone for 2 or 4 hours (h). Autophagy flux was analysed as described in (C). Arrow points to Trim32 specific band; # indicates a non-specific signal. **F)** *Trim32* KO L6E9 cells were obtained by CRISPR-CAS9 using lentiviral vectors. L6E9 cells were cultured in differentiation medium for 3 days and treated, or not, with dexamethasone for 2 or 4 hours (h). Left panel: Autophagy flux was analysed as described in (C). Graph (right panel) reports means ± s.d. of LC3-II/GAPDH values from three independent experiments; * = $p<0.05$.

FIGURE S3

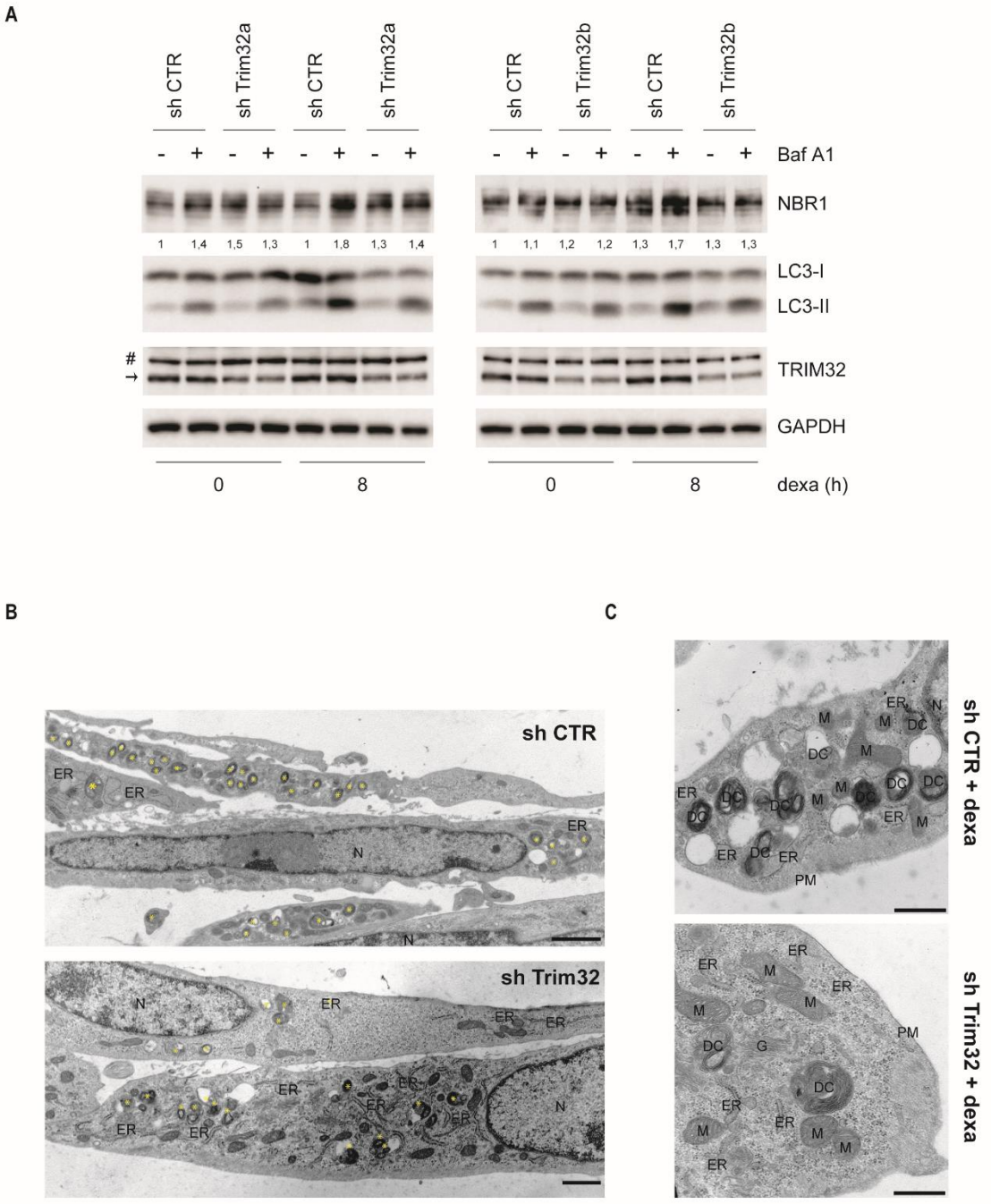


Fig. S3. Analysis of autophagy defects in TRIM32 silenced myoblasts. A) shCTR, shTrim32a (left panel) and shTrim32b (right panel) cells were cultured in differentiation medium for 3 days and incubated with dexamethasone for 8 hours (h). Four hours before lysis, cells were incubated in the presence or absence of the lysosome inhibitor Bafilomycin A1 (Baf A1) to monitor autophagy flux. LC3-II, NBR1 and TRIM32 levels were analysed by immunoblotting. GAPDH was included as a loading control. Arrow points to TRIM32 specific

band; # indicates a non-specific signal. **B)** shCTR and shTrim32 cells, cultured in differentiation medium for 3 days, were processed for electron microscopy as described in Methods. ER, endoplasmic reticulum; N, nucleus. Yellow asterisks highlight degradative compartments (amphisomes, lysosomes and autolysosomes). Size bar, 1 µm. **C)** shCTR and shTrim32 cells, cultured in differentiation medium for 3 days, were treated with dexamethasone (dexa) for 4 hours and processed for electron microscopy. ER, endoplasmic reticulum; N, nucleus; M, mitochondria; G, Golgi apparatus; PM, plasma membrane; DC, degradative compartments (amphisomes, lysosomes and autolysosomes). Size bar, 0,5 µm.

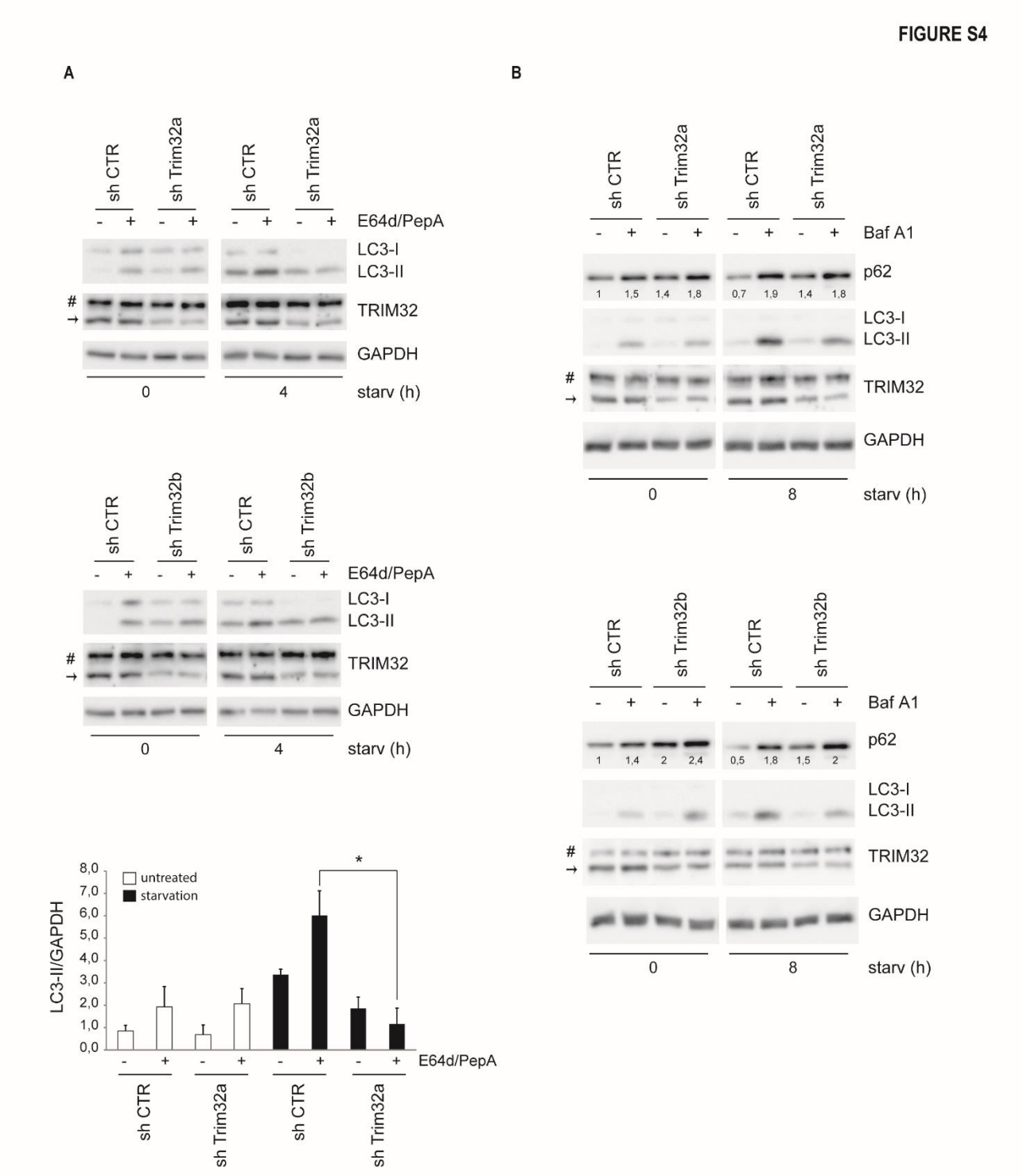
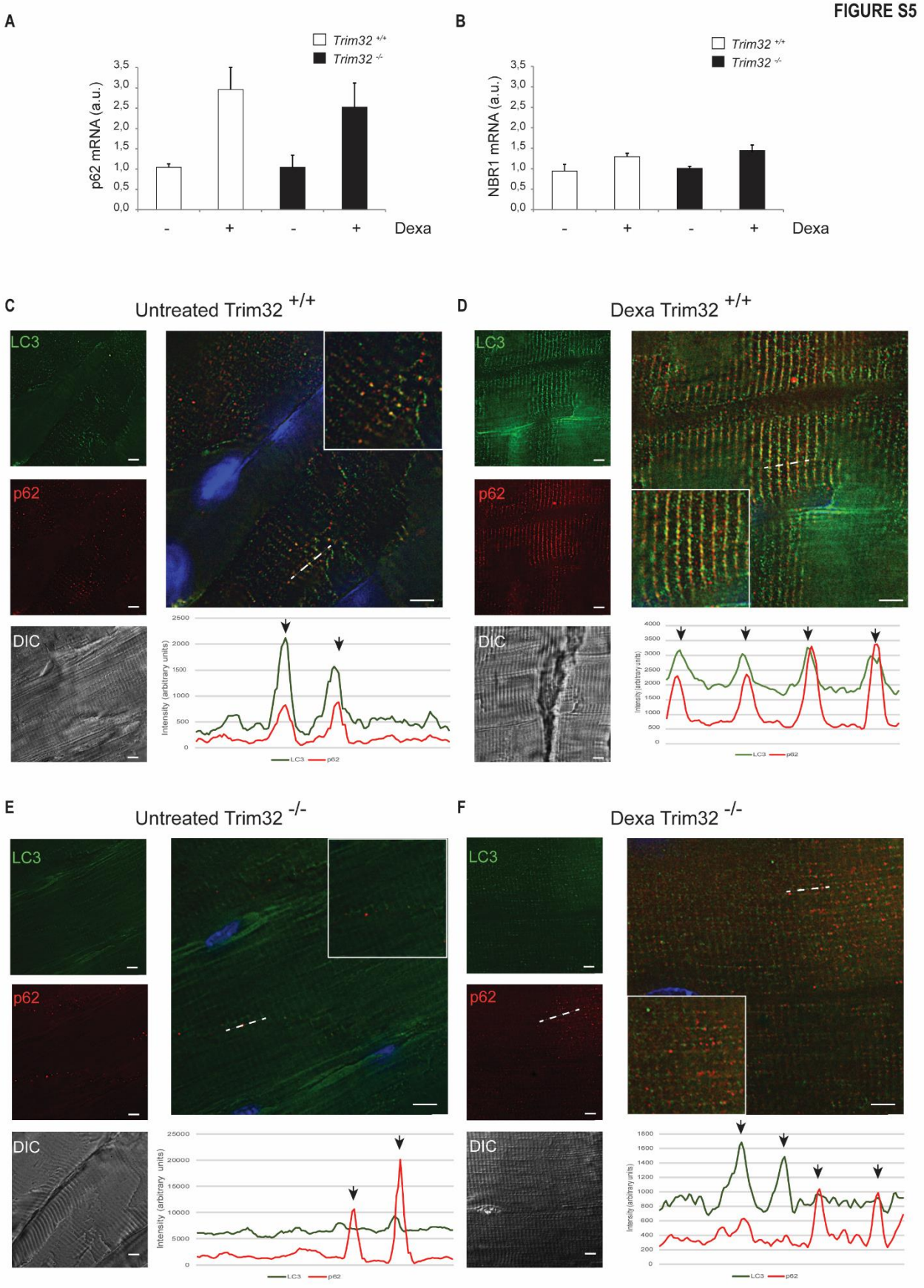


Fig. S4. Role of Trim32 in autophagy induction by nutrient starvation in muscle cells. **A)** shCTR, shTrim32a (upper panel) and shTrim32b (middle panel) cells were differentiated for 3 days and incubated in nutrient-depleted medium (starvation) for 4 hours (h). To monitor autophagy flux, cells were analysed as described in fig. S2C. Arrow points to Trim32 specific band; # indicates a non-specific signal. Graph (lower panel) reports means

± s.d. of LC3-II/GAPDH values from three independent experiments; * = $p<0.05$. **B)** shCTR, shTrim32a (upper panel) and shTrim32b (lower panel) cells were differentiated for 3 days and incubated in nutrient-depleted medium (starvation) for 8 hours (h). Four hours before lysis, cells were incubated in the presence or absence of the lysosome inhibitor Bafilomycin A1 (Baf A1) to monitor autophagy flux. LC3-II, p62 and TRIM32 levels were analysed by immunoblotting. GAPDH was included as a loading control. Arrow point to TRIM32 specific band; # indicates a non-specific signal.

FIGURE S5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Trim32 -/-
p62 mRNA (a.u.)
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2,5
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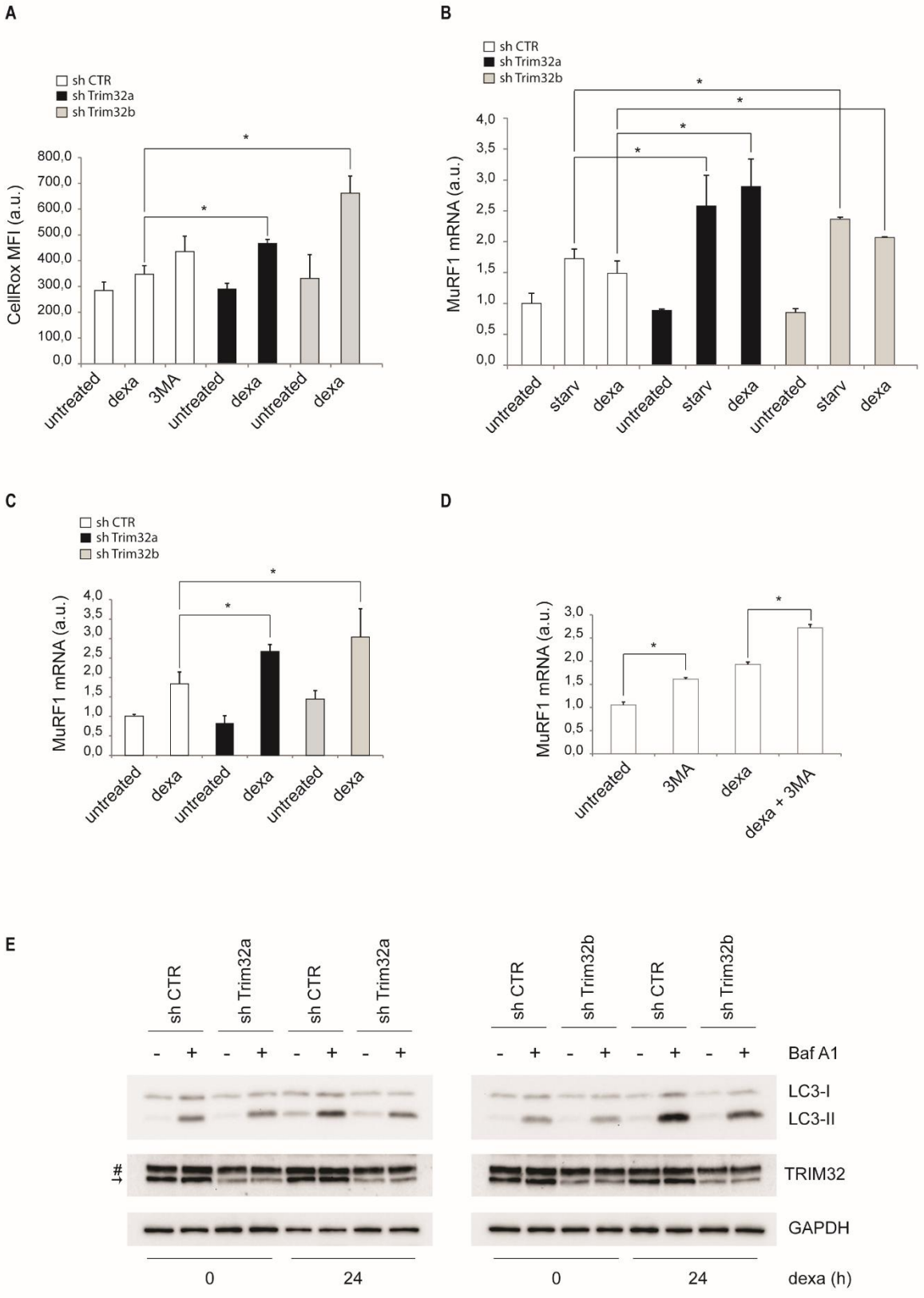
1,5
1,0
0,5
0,0
- + - + Dexa
B
Trim32 +/+
Trim32 -/-
NBR1 mRNA (a.u.)
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
- + - + Dexa
C
Untreated Trim32 +/+
LC3
p62
DIC
Intensity (arbitrary units)
2500
2000
1500
1000
500
0
LC3 p62
D
Dexa Trim32 +/+
LC3
p62
DIC
Intensity (arbitrary units)
4000
3500
3000
2500
2000
1500
1000
500
0
LC3 p62
E
Untreated Trim32 -/-
LC3
p62
DIC
Intensity (arbitrary units)
25000
20000
15000
10000
5000
0
LC3 p62
F
Dexa Trim32 -/-
LC3
p62
DIC
Intensity (arbitrary units)
1800
1600
1400
1200
1000
800
600
400
200
0
LC3 p62

Fig. S5. Analysis of autophagy defects in Trim32$^{-/-}$ mice. A) Real-Time PCR analysis of p62 and NBR1 mRNA levels in quadriceps from Trim32$^{+/+}$ and Trim32$^{-/-}$ mice. Expression levels were normalized on the basis of GAPDH values. A.U.= Arbitrary Unit. Graph reports means ± s.d. of values from 3 independent experiments. **C-F)** LC3 and p62 distribution in fixed and embedded quadriceps from Trim32$^{+/+}$ and Trim32$^{-/-}$ mice by immunofluorescence microscopy. Hoechst dye was used to stain for the nuclei. Single focal plane fluorescence and bright-field images are shown. Tissues from a total of 4 mice per group were examined. Representative images of quadriceps from a Trim32$^{+/+}$ and Trim32$^{-/-}$ animal treated with saline (C and E, respectively) or with Dexamethasone for 24 hours (D and F, respectively) are presented. The degree of co-localization between LC3- and p62-positive puncta in each sample is also highlighted by generating a fluorescence intensity profile using the Icy software (Institut Pasteur), in the region of each merged picture marked with a dash line. Scale bars, 5 µm.

FIGURE S6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300,0
200,0
100,0
0,0
untreated
dexa
3MA
untreated
dexa
untreated
dexa
B
sh CTR
sh Trim32a
sh Trim32b
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2,0
1,5
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0,5
0,0
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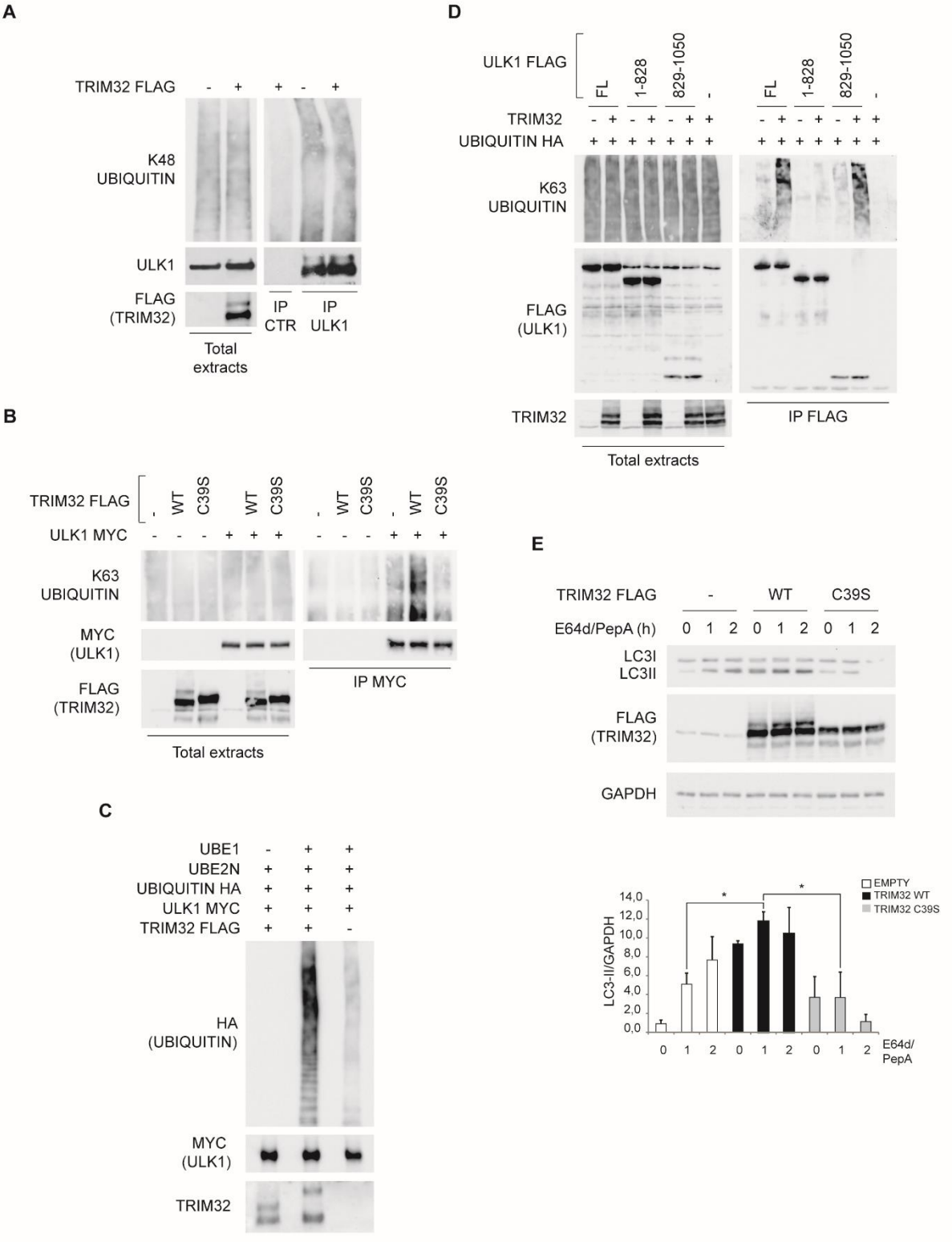
starv
dexa
untreated
starv
dexa
untreated
starv
dexa
C
sh CTR
sh Trim32a
sh Trim32b
MuRF1 mRNA (a.u.)
untreated
dexa
untreated
dexa
untreated
dexa
D
MuRF1 mRNA (a.u.)
untreated
3MA
dexa
dexa + 3MA
E
sh CTR
sh Trim32a
sh CTR
sh Trim32a
sh CTR
sh Trim32b
sh CTR
sh Trim32b
Baf A1
LC3-I
LC3-II
TRIM32
GAPDH
0
24
0
24
dexa (h)

Fig. S6. Analysis of MuRF1 and ROS levels in dexamethasone-treated muscle cells. **A)** CellROX staining in shCTR, shTrim32a and shTrim32b cells incubated for 24 hours with dexamethasone or 3MA (only in shCTR). MFI= Mean fluorescent intensity, A.U.= Arbitrary Unit. Graph reports means ± s.d. of values from 3 independent experiments. **B)** Real-Time PCR analysis of MuRF1 mRNA levels in shCTR, shTrim32a and shTrim32b cells incubated for 8 hours with dexamethasone (dexa) or with nutrient deprived medium (starv). Expression levels were normalized on the basis of GAPDH values. A.U.= Arbitrary Unit. Graph reports means ± s.d. of values from 3 independent experiments. **C)** Real-Time PCR analysis of MuRF1 mRNA levels in shCTR, shTrim32a and shTrim32b cells incubated for 24 hours with dexamethasone. Expression levels were normalized on the basis of GAPDH values. A.U.= Arbitrary Unit. Graph reports means ± s.d. of values from 3 independent experiments. **D)** Real-Time PCR analysis of MuRF1 mRNA levels in C2.7 cells incubated for 24 hours with dexamethasone and the autophagy inhibitor 3-methyladenine (3MA), individually or in combination. Expression levels were normalized on the basis of GAPDH values. A.U.= Arbitrary Unit. Graph reports means ± s.d. of values from 3 independent experiments. **E)** shCTR, shTrim32a (left panel) and shTrim32b (right panel) cells were cultured in differentiation medium for 3 days and incubated with dexamethasone for 24 hours (h). Eight hours before lysis, cells were incubated in the presence or absence of the lysosome inhibitor Bafilomycin A1 (Baf A1) to monitor autophagy flux. LC3-II and TRIM32 levels were analysed by immunoblotting. GAPDH was included as a loading control. Arrow points to TRIM32 specific band; # indicates a non-specific signal.

FIGURE S7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ULK1
FLAG (TRIM32)
IP CTR
IP ULK1
Total extracts
B
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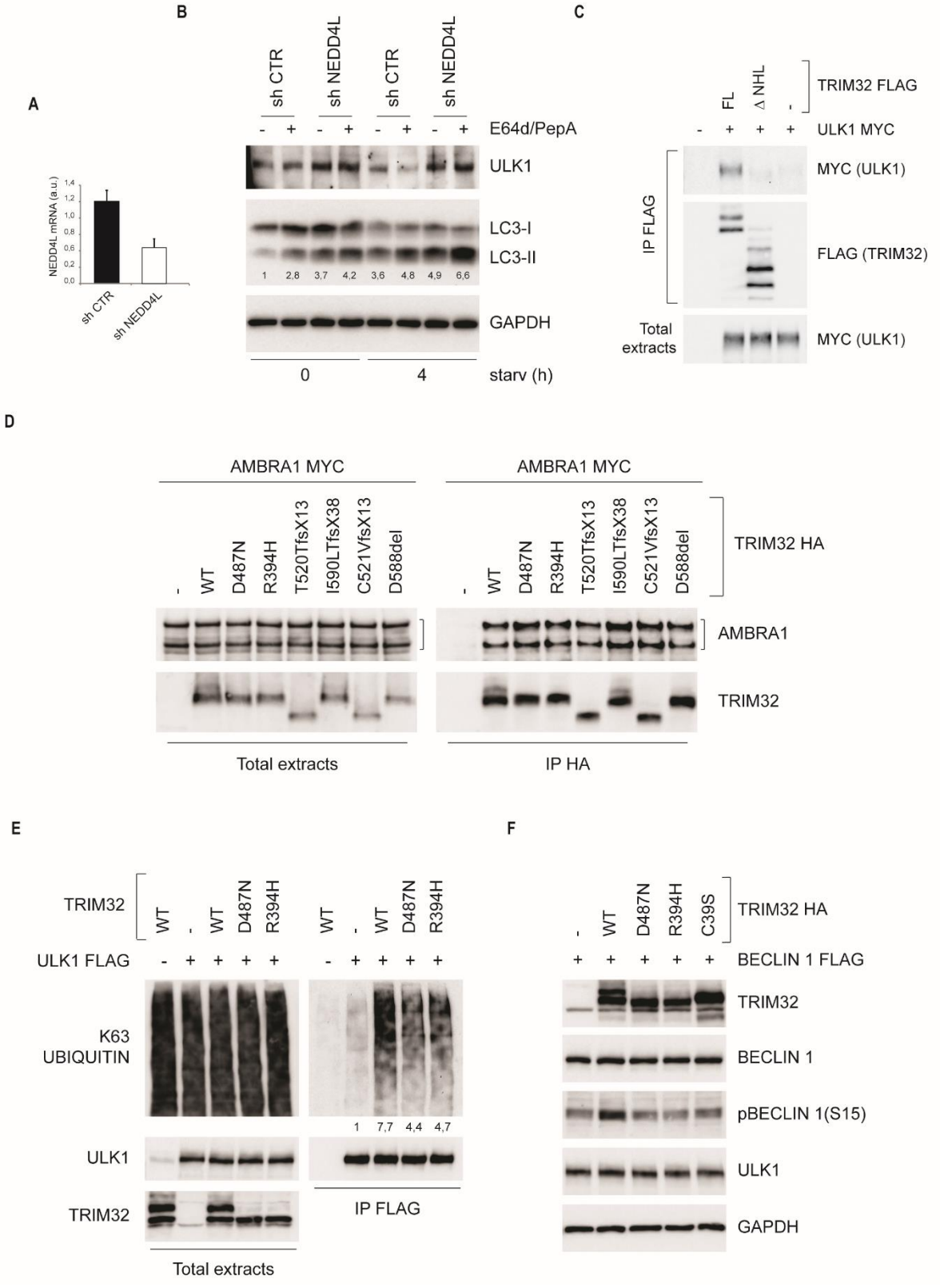
WT
C39S
ULK1 MYC
K63 UBIQUITIN
MYC (ULK1)
FLAG (TRIM32)
IP MYC
Total extracts
C
UBE1
UBE2N
UBIQUITIN HA
ULK1 MYC
TRIM32 FLAG
HA (UBIQUITIN)
MYC (ULK1)
TRIM32
D
ULK1 FLAG
FL
1-828
829-1050
TRIM32
UBIQUITIN HA
K63 UBIQUITIN
FLAG (ULK1)
TRIM32
IP FLAG
Total extracts
E
TRIM32 FLAG
WT
C39S
E64d/PepA (h)
LC3I
LC3II
FLAG (TRIM32)
GAPDH
EMPTY
TRIM32 WT
TRIM32 C39S
LC3-II/GAPDH
E64d/ PepA

Fig. S7. Characterization of the proautophagic properties of TRIM32. A) 293T cells were transfected with a vector encoding FLAG-TRIM32, or an empty vector as negative control. Protein extracts were subjected to immunoprecipitation using an anti ULK1 antibody. Immunopurified complexes were analysed by immunoblotting to detect the presence of ULK1 and K48-linked ubiquitin. Total extracts were also probed with an anti-TRIM32 antibody to verify transfection efficiency. **B)** 293T cells were transfected with vectors encoding HA-tagged ubiquitin and MYC-ULK1, alone or in combination with vectors for FLAG TRIM32 WT or the catalytic mutant C39S. Protein extracts were subjected to immunoprecipitation using an anti-MYC antibody. Immunopurified complexes were analysed by immunoblotting to detect the presence of K63-linked ubiquitin using a specific antibody, and ULK1 using an anti-MYC antibody. Total extracts were also probed with an anti-FLAG antibody to verify transfection efficiency. **C)** In vitro ULK1 ubiquitination assay using FLAG TRIM32 and MYC-ULK1 immunopurified from 293T cells, and recombinant HA Ubiquitin, E1 Ub-activating enzyme (UBE1) and E2 Ub-conjugating enzyme (UBE2N). Reactions were also performed in absence of either the E1 Ub-activating enzyme or TRIM32 to verify ubiquitination reaction specificity. At the end of the reaction, ULK1 was immunopurified using a specific antibody, and its ubiquitination was evaluated by immunoblotting using an anti-HA antibody. **D)** 293T cells were transfected with vectors encoding HA-tagged ubiquitin together with one of the following FLAG-tagged constructs: ULK1 full length, ULK1 aa 1-828, ULK1 aa 829-1050. Cells were subsequently transfected with an expression vector for TRIM32 or an empty vector. Protein extracts were subjected to immunoprecipitation using an anti-FLAG antibody. Immunopurified complexes were analysed by immunoblotting to detect the presence of K63-linked ubiquitin and ULK1, using specific antibodies. Total extracts were also probed with an anti-TRIM32 antibody to verify transfection efficiency. **E)** 293T cells were transfected with vectors encoding for FLAG-TRIM32 WT or the catalytic mutant C39S. Forty-eight hours after transfection, cells were incubated with lysosome inhibitors E64d and pepstatin A for 1 or 2 hours (h), or left untreated. Cells were lysed and the levels of LC3 and TRIM32 were analyzed by immunoblotting. GAPDH was included as a loading control (upper panel). Graph (lower panel) reports means ± s.d. of LC3-II/GAPDH values from three independent experiments; * = $p<0.05$.

FIGURE S8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sh NEDD4L
B
sh CTR
sh NEDD4L
sh CTR
sh NEDD4L
E64d/PepA
ULK1
LC3-I
LC3-II
1 2,8 3,7 4,2 3,6 4,8 4,9 6,6
GAPDH
0
4
starv (h)
C
FL
Δ NHL
-
TRIM32 FLAG
ULK1 MYC
IP FLAG
MYC (ULK1)
FLAG (TRIM32)
Total extracts
MYC (ULK1)
D
AMBRA1 MYC
AMBRA1 MYC
-
WT
D487N
R394H
T520TfsX13
I590LTfsX38
C521VfsX13
D588del
TRIM32 HA
AMBRA1
TRIM32
Total extracts
IP HA
E
TRIM32
ULK1 FLAG
K63 UBIQUITIN
1 7,7 4,4 4,7
ULK1
TRIM32
IP FLAG
Total extracts
F
C39S
TRIM32 HA
BECLIN 1 FLAG
TRIM32
BECLIN 1
pBECLIN 1(S15)
ULK1
GAPDH

Fig. S8. Regulation of ULK1 activity by NEDD4L and TRIM32. A) Real-Time PCR analysis of NEDD4L mRNA levels in shCTR and shNEDD4L C2.7 cells. Expression levels were normalized on the basis of GAPDH values. A.U.= Arbitrary Unit. Graph reports means ± s.d. of values from 3 independent experiments. **B)** shCTR and shNEDD4L C2.7 cells were differentiated for 3 days and incubated in nutrient-depleted medium (starv) for 4 hours (h). Two hours before lysis, cells were incubated with the lysosome inhibitors E64d and Pepstatin A (E64d/PepA) to monitor autophagy flux, as indicated. LC3-II and ULK1 levels were analysed by immunoblotting. GAPDH was included as a loading control. **C)** 293T cells were transfected with vectors encoding MYC-ULK1 in combination with FLAG-TRIM32 full length (FL) or FLAG-TRIM32 mutant lacking the NHL repeats (ΔNHL: aa 1-326). Protein extracts were immunoprecipitated using an anti-FLAG antibody. Immunopurified complexes were analysed by immunoblotting using anti-MYC and anti-FLAG antibodies to detect the presence of ULK1 and TRIM32, respectively. **D)** 293T cells were transfected with a vector encoding for MYC-AMBRA1 alone or in combination with HA-TRIM32 wild-type (WT) or the indicated pathogenic TRIM32 mutants. Cells were lysed 48 hours after transfection and protein extracts subjected to immunoprecipitation using an anti-HA antibody. Immunopurified complexes were analysed by immunoblotting to detect the presence of TRIM32 and AMBRA1. **E)** 293T cells were transfected with vectors encoding TRIM32 WT, D487N, R394H in combination with ULK1 FLAG and ubiquitin HA. Protein extracts were subjected to immunoprecipitation using an anti-FLAG antibody. Immunopurified complexes were analysed by immunoblotting to detect the presence of ULK1 and K63-linked ubiquitin. Total extracts were also probed with an anti TRIM32 antibody to verify TRIM32 transfection. **F)** 293T cells were transfected with vectors encoding HA-tagged TRIM32 WT, D487N, R394H, C39S in combination with BECLIN 1 FLAG. Protein extracts were analysed by immunoblotting to detect the levels of TRIM32, BECLIN 1, phospho-BECLIN 1 (Ser15) and ULK1. GAPDH was included as a loading control.

FIGURE S9

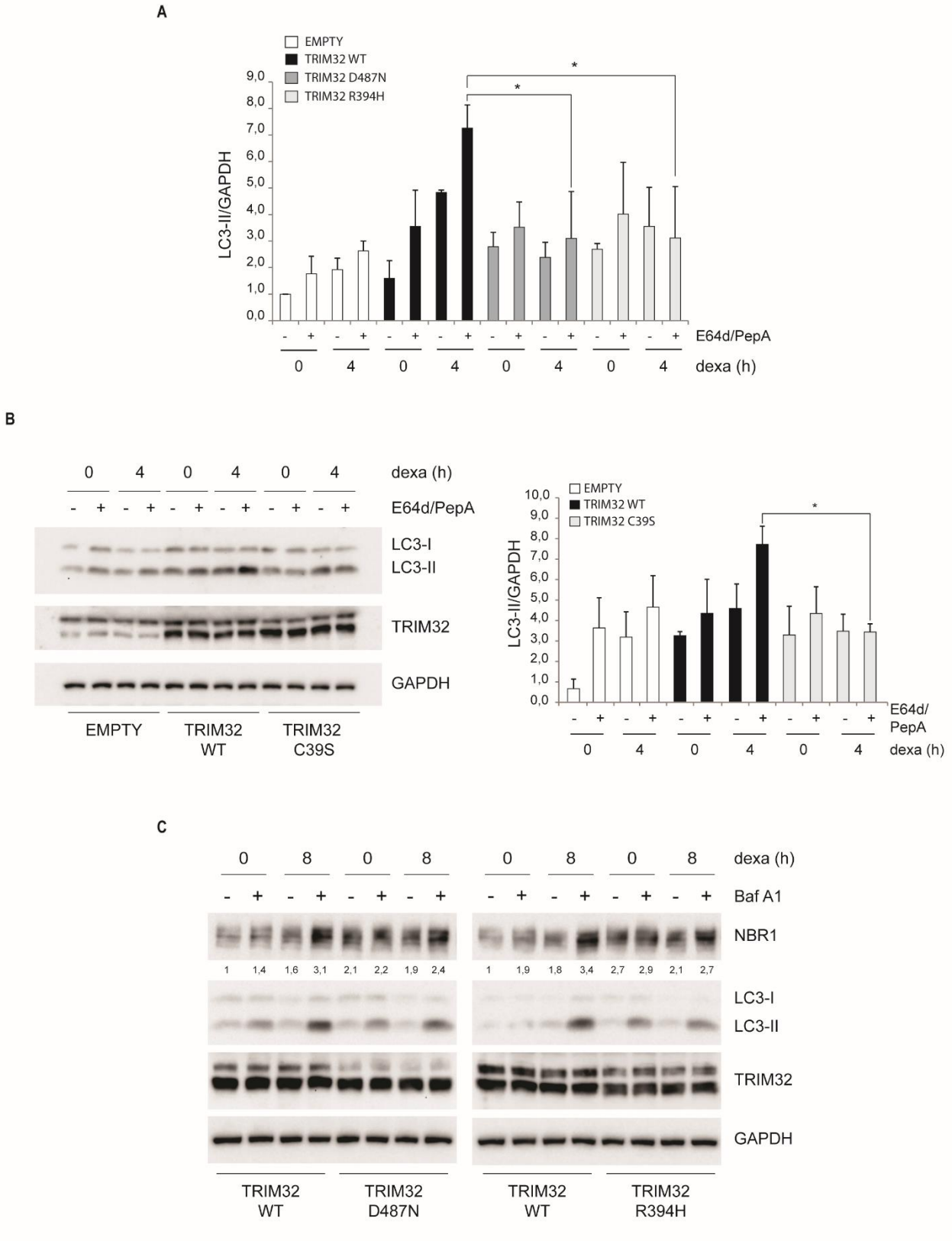
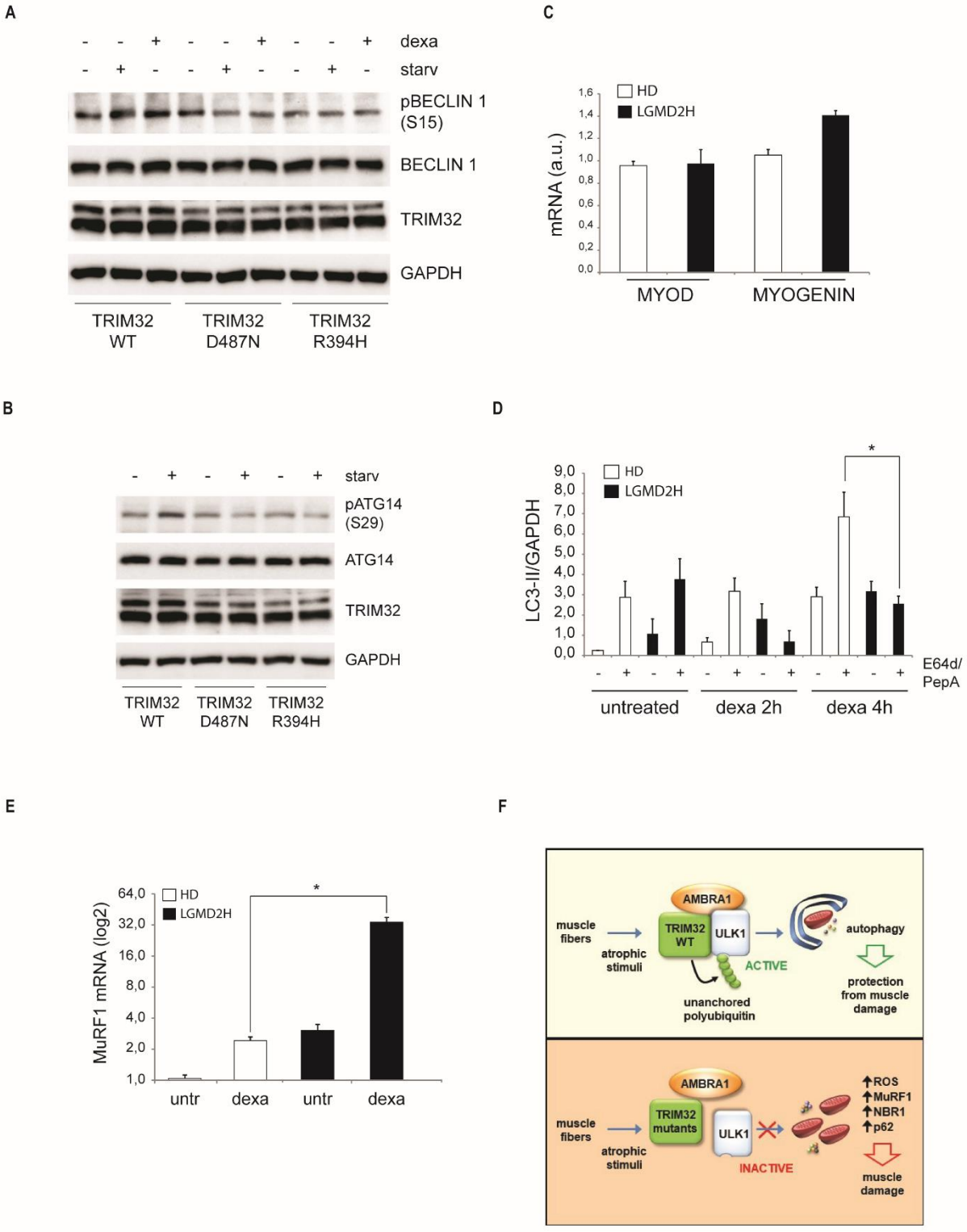

A
EMPTY
TRIM32 WT
TRIM32 D487N
TRIM32 R394H
LC3-II/GAPDH
E64d/PepA
dexa (h)
B
dexa (h)
E64d/PepA
LC3-I
LC3-II
TRIM32
GAPDH
EMPTY
TRIM32 WT
TRIM32 C39S
TRIM32 C39S
E64d/ PepA
C
dexa (h)
Baf A1
NBR1
LC3-I
LC3-II
TRIM32
GAPDH
TRIM32 WT
TRIM32 D487N
TRIM32 WT
TRIM32 R394H

Fig. S9. Characterization of autophagy properties of C2.7 cells expressing TRIM32 pathological mutants. A) Quantification of LC3-II levels in *Trim32* KO C2.7 cells complemented with the Trim32 pathogenic mutants, shown in Fig. 6B. Graph reports means ± s.d. of LC3-II/Gapdh values from 3 independent experiments; * = $p<0.05$. **B)** *TRIM32* KO C2.7 were infected with retroviruses encoding TRIM32 WT or the catalytic mutant C39S. EMPTY: *TRIM32* KO C2.7 infected with non-coding retroviruses. Upon 3 days in differentiation medium, cells were treated with dexamethasone for 4 hours (h), and incubated with the lysosome inhibitors E64d and pepstatin A (E64d/PepA) for 1 hour before lysis, as indicated. Left panel: LC3-II and TRIM32 levels were analyzed by immunoblotting using specific antibodies. GAPDH was included as a loading control. Right panel: graph reporting means ± s.d. of LC3-II/Gapdh values from 3 independent experiments; * = $p<0.05$. **C)** *TRIM32* KO C2.7 were infected with retroviruses encoding WT FLAG-TRIM32 or the indicated pathogenic TRIM32 mutants. Upon 3 days in differentiation medium, cells were treated with dexamethasone for 8 hours (h), and incubated with the lysosome inhibitor Bafilomycin A1 (Baf1) for 4 hour before lysis, as indicated. NBR1, LC3-II and TRIM32 levels were analyzed by immunoblotting using specific antibodies. GAPDH was included as a loading control.

FIGURE S10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dexa
starv
pBECLIN 1 (S15)
BECLIN 1
TRIM32
GAPDH
TRIM32 WT
TRIM32 D487N
TRIM32 R394H
C
HD
LGMD2H
mRNA (a.u.)
MYOD
MYOGENIN
B
starv
pATG14 (S29)
ATG14
TRIM32
GAPDH
TRIM32 WT
TRIM32 D487N
TRIM32 R394H
D
HD
LGMD2H
LC3-II/GAPDH
E64d/ PepA
untreated
dexa 2h
dexa 4h
E
HD
LGMD2H
MuRF1 mRNA (log2)
untr
dexa
untr
dexa
F
muscle fibers
atrophic stimuli
AMBRA1
TRIM32 WT
ULK1
ACTIVE
unanchored polyubiquitin
autophagy
protection from muscle damage
AMBRA1
TRIM32 mutants
ULK1
INACTIVE
ROS
MuRF1
NBR1
p62
muscle damage

Fig. S10. Defective autophagy induction in human and murine myoblast cells expressing TRIM32 pathological mutants. A) *TRIM32* KO C2.7 overexpressing human BECLIN 1 were infected with retroviruses encoding FLAG-TRIM32 WT or the indicated pathogenic TRIM32 mutants. Cells were differentiated for 3 days and incubated with dexamethasone or nutrient deprived medium for 2 hours. Protein extracts were analysed by immunoblotting to measure the levels of phospho-BECLIN 1 (Ser15), BECLIN 1 and TRIM32. GAPDH was included as a loading control. **B)** *TRIM32* KO C2.7 were infected with retroviruses encoding FLAG-TRIM32 WT or the indicated pathogenic TRIM32 mutants. Cells were cultured in differentiation medium for 3 days and incubated with dexamethasone for 2 hours. Cells were lysed and protein extracts were analysed by immunoblotting to measure the levels of phospho-ATG14 (Ser29), ATG14 and TRIM32. GAPDH was included as a loading control. **C)** Fibroblasts from a wild type donor and a LGMD2H patient were transdifferentiated to myoblasts by MYOD ectopic expression. Expression levels of MYOD and MYOGENIN were evaluated by RT-PCR. Expression levels were normalized on the basis of GAPDH values (a.u.: arbitrary units). **D)** Quantification of LC3II levels in cells from a LGMD2H patient or a normal control, treated as described in Fig. 6C. Graph reports means ± s.d. of LC3-II/GAPDH values from three independent experiments; * = $p<0.05$. **E)** Real-Time PCR analysis of MuRF1 mRNA levels in cells from a wild type donor and a LGMD2H patient incubated for 24 hours with dexamethasone. Expression levels were normalized on the basis of GAPDH values. Data are shown on a log 2 scale. Graph reports means ± s.d. of values from 3 independent experiments. * = $p<0.05$. **F)** The ubiquitin ligase TRIM32 regulates autophagy under atrophic conditions. Top: In wild type cells, TRIM32 binds AMBRA1 and ULK1, and activate ULK1 via unanchored polyubiquitin. Autophagy induction contributes to the survival of muscle fibers in atrophic conditions. Bottom: LGMD2H-causative mutations impair TRIM32 ability to bind ULK1 and to induce autophagy. Defective autophagy leads to the accumulation of ROS, impaired degradation of p62 and NBR1, and increased expression of MuRF1.