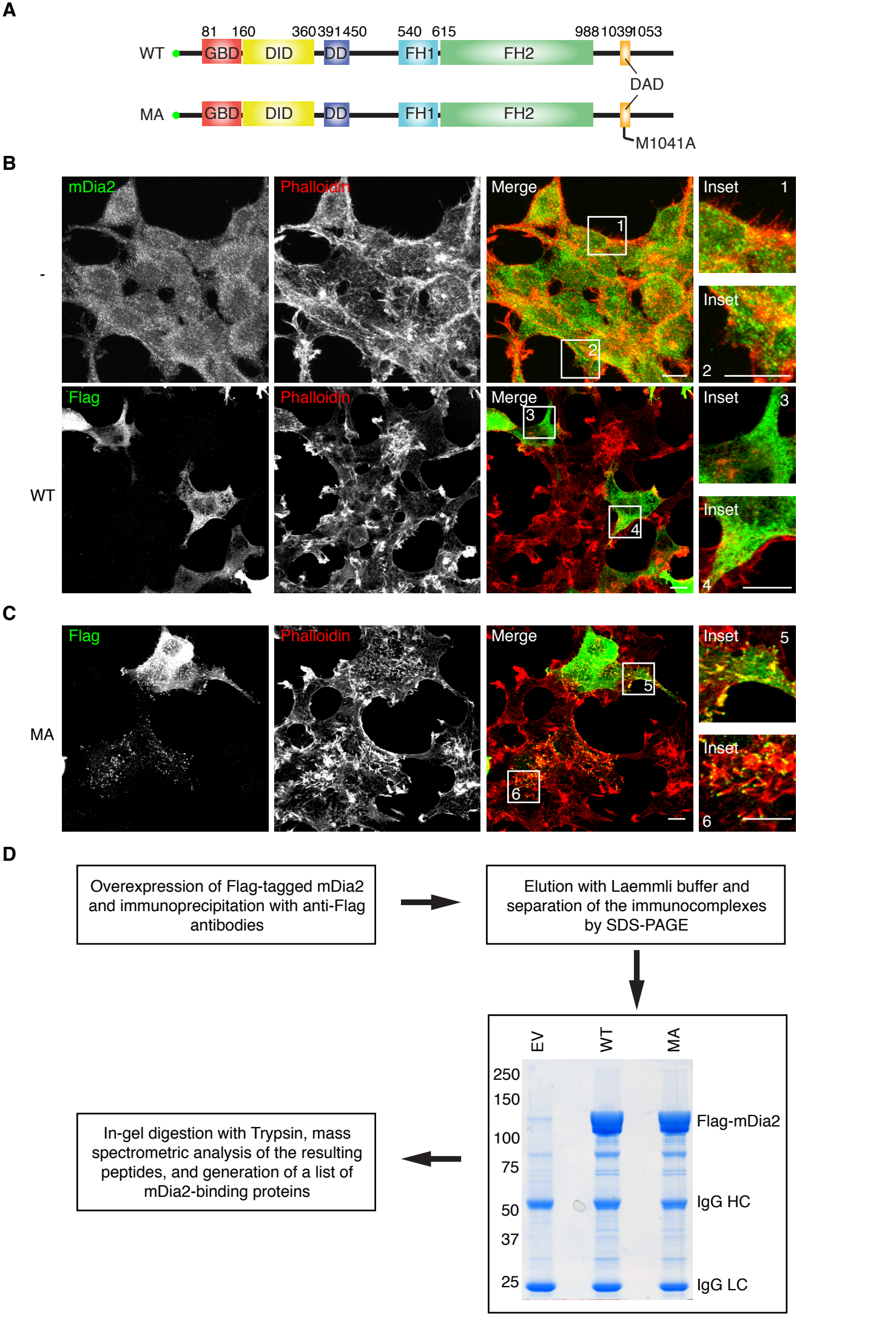
Supplemental Figure 1

(**A**) Schematic of wild-type mDia2 and its M1041A mutant. Domains and motifs in the primary sequence of *Mus musculus* mDia2 (wild-type mDia2 = WT) are depicted as coloured boxes and their amino acid boundaries indicated on the top. The M1041-to-A point mutation is highlighted in the lower drawing (mDia2 M1041A = MA). GBD: GTPase binding domain; DID: Diaphanous-inhibitory domain; DD: dimerization domain; FH: Formin homology domain; DAD: Diaphanous auto-inhibitory domain. Flag tag is depicted as a filled green circle (not in scale). (**B**) Localization of endogenous and Flag-tagged wild-type mDia2. *Top row*: 293T cells were fixed and stained with anti-mDia2 antibodies (mDia2, green) and Rhodamine phalloidin (Phalloidin, red) to detect mDia2 and F-actin, respectively. *Bottom row*: 293T cells were transfected with Flag-tagged wild-type mDia2 (WT), fixed and stained with anti-Flag antibodies (Flag, green) and Rhodamine phalloidin (Phalloidin, red) to detect mDia2 and F-actin, respectively. Insets zoom in to the localization of endogenous and wild-type mDia2. Scale bars are 10 μm. (**C**) Localization of constitutively active mDia2. 293T cells were transfected with Flag-tagged mDia2 M1041A (MA), fixed and stained with anti-Flag antibodies (Flag, green) and Rhodamine phalloidin (Phalloidin, red) to detect mDia2 and F-actin, respectively. Insets zoom in to the localization of mDia2 MA at the tip of filopodia. Scale bars are 10 μm. (**D**) Isolation of the mDia2 interactome: flowchart and typical results. Flag-tagged mDia2 (either WT or MA), or the corresponding empty vector (EV), were overexpressed in 293T cells, immunoprecipitated and mDia2-based complexes processed as described in the Methods. Proteins were in-gel digested with Trypsin and the resulting peptides subjected to mass spectrometry to compile a list of mDia2-binding proteins.

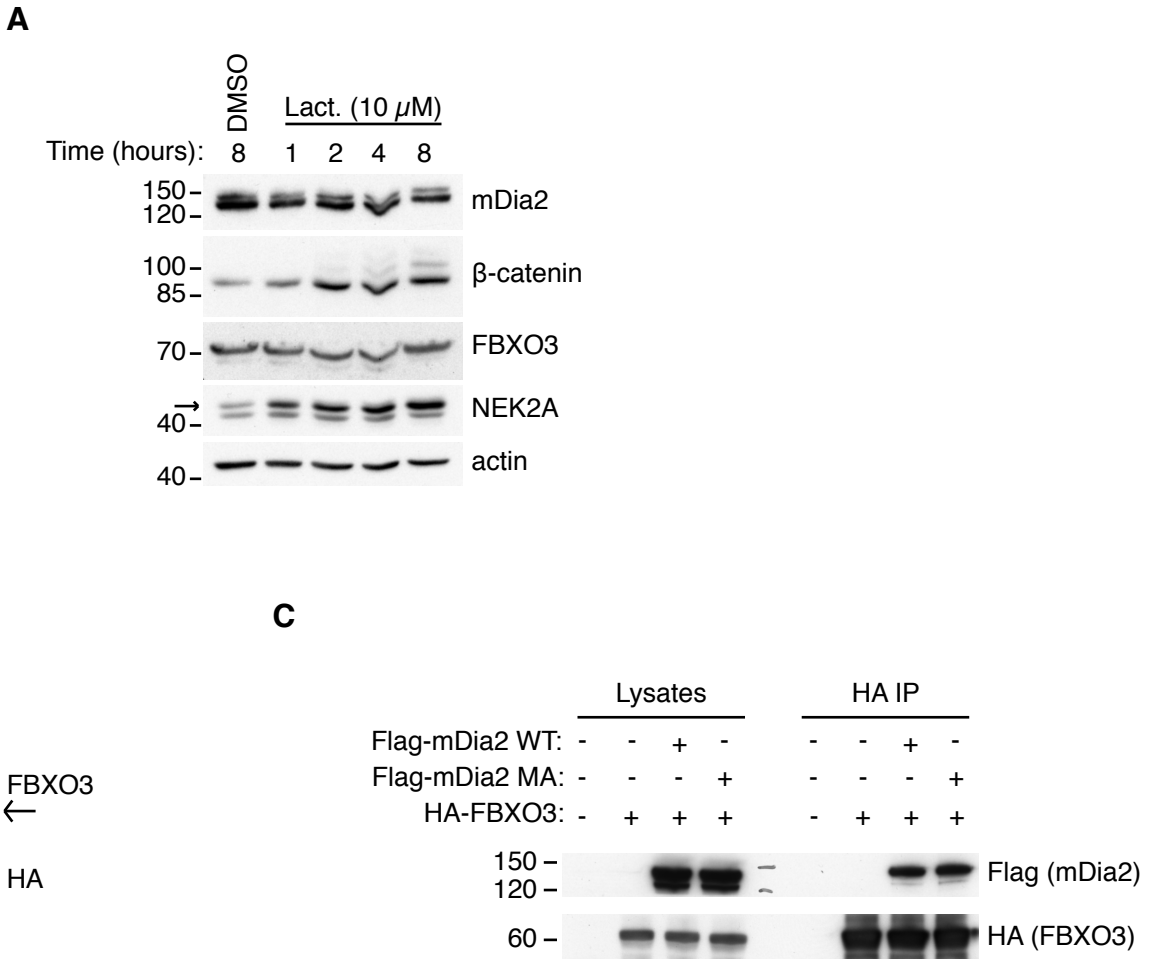


Supplemental Figure 1

Supplemental Figure 2

(**A**) mDia2 is not a proteasome substrate. 293T cells were treated with DMSO or Lactacystin (Lact.) (10 μM) for the indicated time prior to lysis. Total cell lysates (20 μg) were separated by SDS-PAGE and immunoblotted with the indicated antibodies. β-catenin and NEK2A, two genuine proteasome substrates, served as control for the effectiveness of the Lactacystin treatment. Actin served as loading control. Arrow marks endogenous NEK2A in the anti-NEK2A blot, which also show a non-specific cross-reacting band. (**B**) HA-tagged FBXO3 is less expressed than its endogenous counterpart. 293T cells were transfected with HA-tagged FBXO3 (+) or the corresponding empty vector (-) (10 μg in either case). Total cell lysates (30 μg) were separated by SDS-PAGE and the expression of endogenous and HA-tagged FBXO3 assessed by immunoblotting with anti-FBXO3 and anti-HA antibodies, respectively. Arrow marks HA-FBXO3 in the anti-FBXO3 blot. The migration of endogenous FBXO3 is retarded most likely by its ubiquitination. One of two experiments that were performed with similar results is shown. (**C**) FBXO3 binds similarly to both wild-type and constitutively active mDia2. 293T cells were co-transfected with equal amounts (5 μg) of Flag-tagged mDia2 (WT or MA) and HA-tagged FBXO3 (+) or the corresponding empty vectors (-), in the combinations indicated on top. Anti-HA immunoprecipitations (HA IP) were performed as in Fig. 3b. Lysate (2%) and immunocomplexes (IP) were separated by SDS-PAGE and immunoblotted with anti-Flag and anti-HA antibodies to detect mDia2 and FBXO3, respectively. One of two experiments that were performed with similar results is shown. (**D**) Flag-tagged p53, HA-tagged FBXO3 and myc-tagged mDia2 expression levels. Total lysates (30 μg) of Fig. 3e were separated by SDS-PAGE and immunoblotted as indicated.

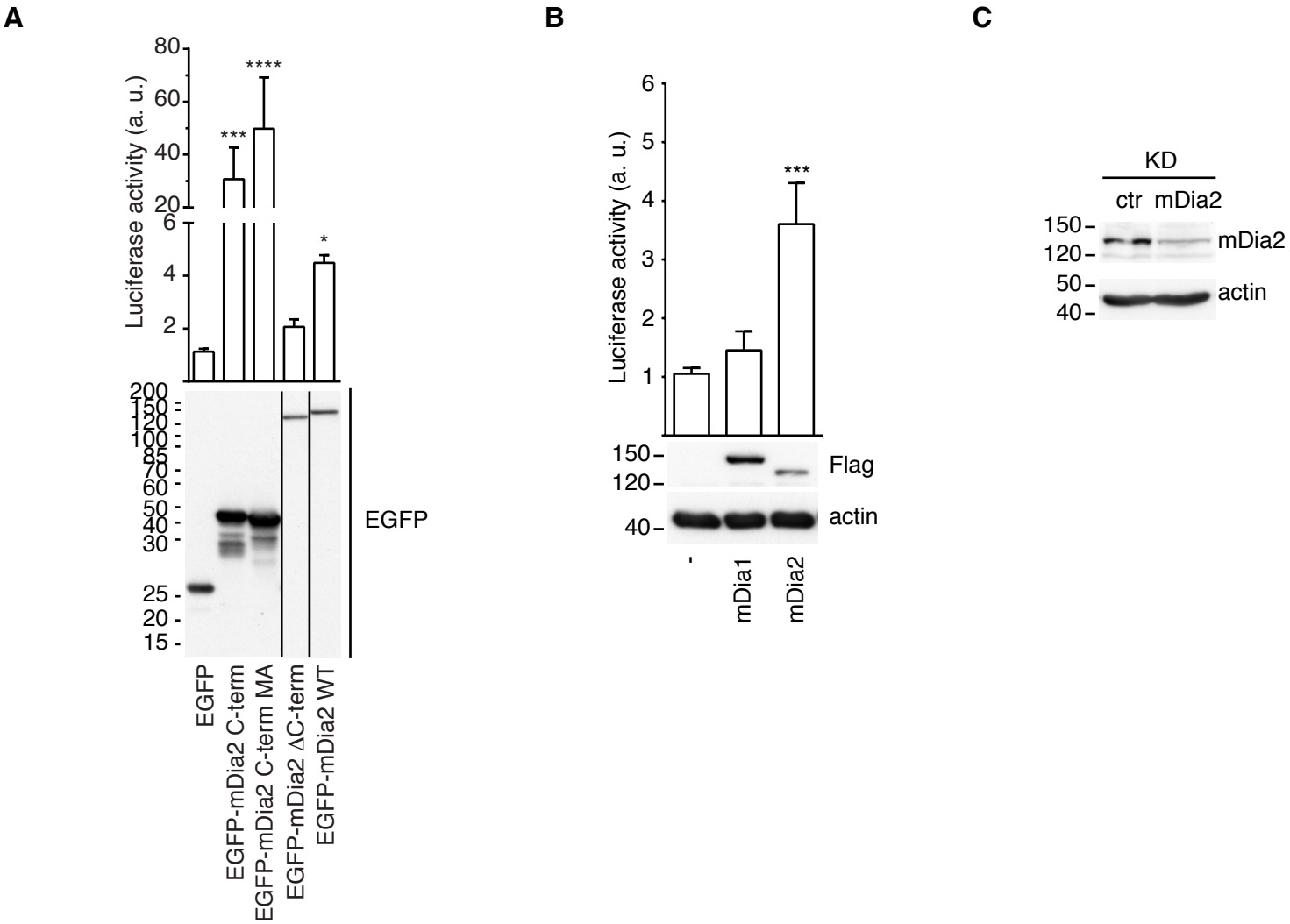
HA-FBXO3 is below the detection limit in the anti-FBXO3 blot. Flag-tagged p53 corresponds to the higher band detected by the anti-p53 antibody.



Supplemental Figure 2

Supplemental Figure 3

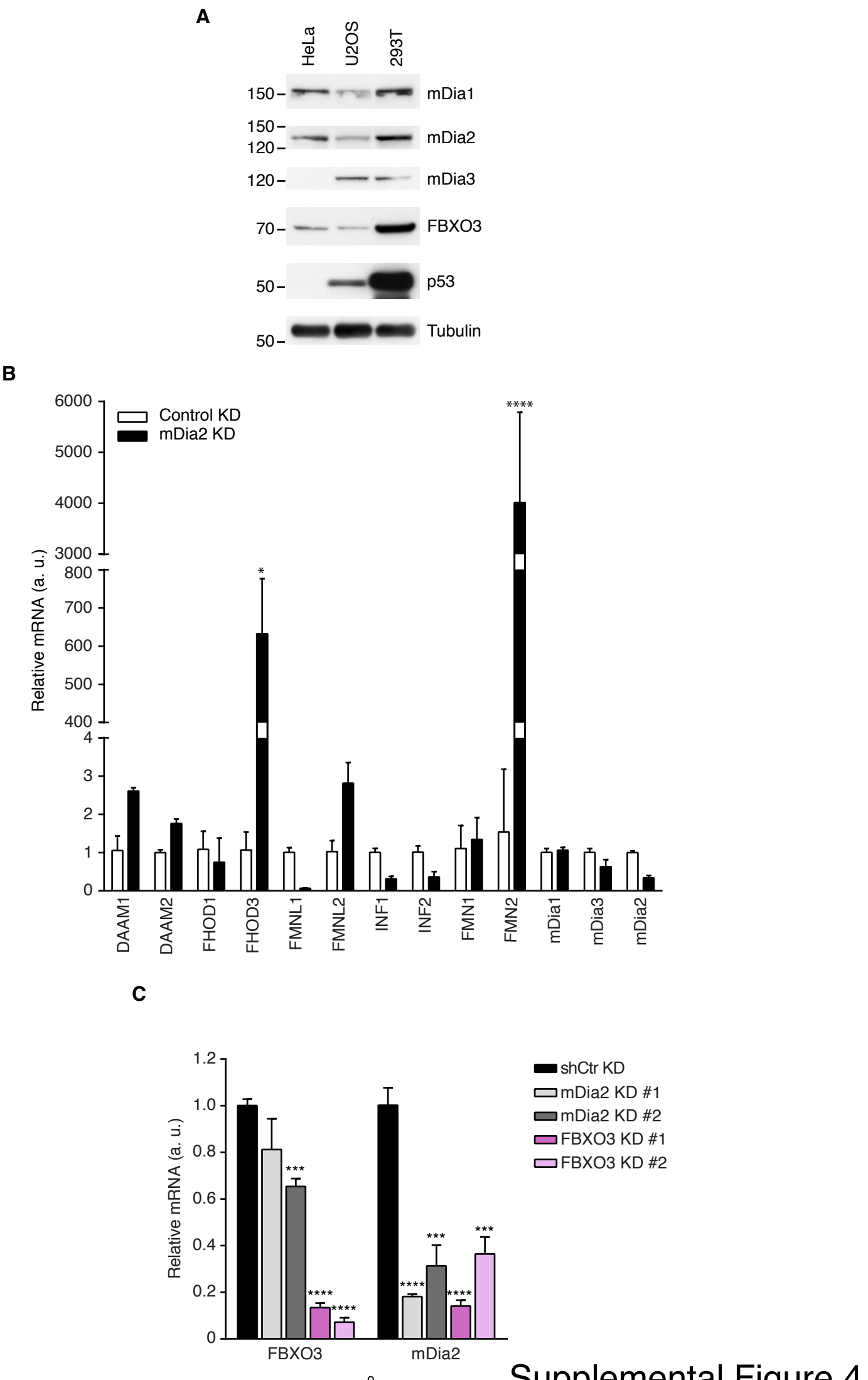
(**A**) The C-terminus of mDia2 is necessary and sufficient to stimulate p53 transcriptional activity. 293T cells were co-transfected with the HMD2 reporter plasmid and either empty vector (EGFP), or EGFP-tagged wild-type full-length mDia2 (EGFP-mDia2 WT) and deletion mutants thereof. Domain boundaries are indicated in Fig. 2a. Luciferase activity (arbitrary units, a.u.) was measured and plotted as described in the Experimental Procedures. Data represent mean ± SD (n = 7, One-way ANOVA). EGFP and mDia2 expression was confirmed using anti-EGFP antibodies and actin served as a loading control. Black lines mark the removal of intervening lanes. (**B**) mDia1 does not activate p53. 293T cells were co-transfected with the HDM2-luciferase reporter plasmid along with the empty vector (-), Flag-tagged wild-type mDia1 (mDia1) or mDia2 (mDia2). The Luciferase activity (arbitrary units, a.u.) measured and plotted as described in the Experimental Procedures. Data represent mean ± SD (n = 9, One-way ANOVA). The expression of mDia1 and mDia2 was checked by immunoblotting with anti-Flag antibodies. Actin served as a loading control. (**C**) RNAi of mDia2 in 293T cells results in a partial knockdown. Control knockdown (ctr) and mDia2 knockdown (mDia2) cells were generated using siRNAs, as described in the Experimental Procedures. Total lysates (30 μg) were separated by SDS-PAGE and immunoblotted as indicated. One of two experiments that were performed with similar results is shown.



Supplemental Figure 3

Supplemental Figure 4

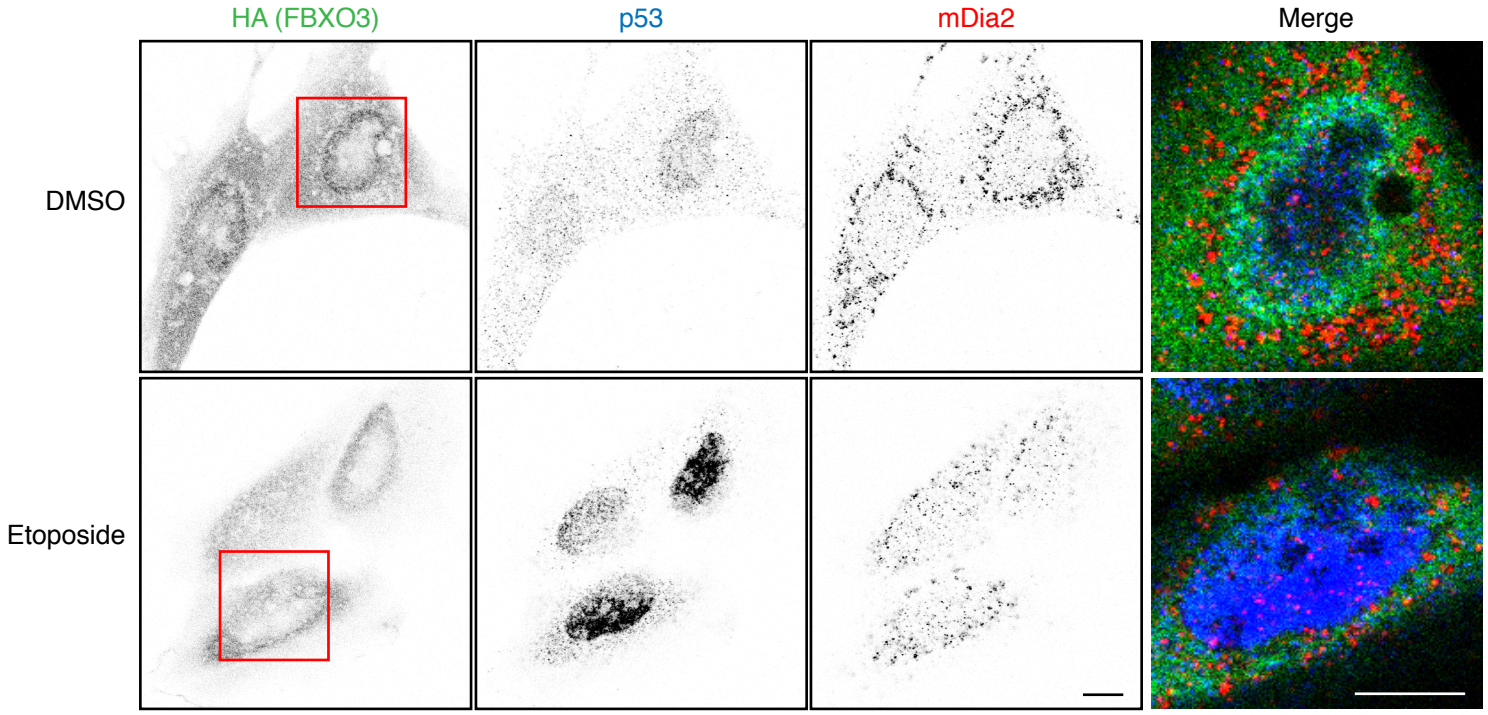
(**A**) Side-to-side comparison of the protein levels of mDia1-3, FBXO3 and p53 in HeLa, U2OS and 293T cells. The cell lines indicated on the top were lysed as described in the Experimental Procedures. Total lysates (30 μg) were separated by SDS-PAGE and immunoblotted as indicated. Tubulin served as a loading control. One of two experiments that were performed with similar results is shown. (**B**) Formin expression landscape in control and mDia2 knockdown cells. mDia2 silencing in U2OS cells was achieved by RNAi as described for HeLa cells (Beli et al., 2008). Total RNA was obtained from both control (Control KD) and mDia2 knockdown (mDia2 KD) cells as indicated in Figure 4c. RT-qPCR analyses were carried out using primers specific for the indicated Formin and cyclophilin as a normalizing gene. The relative levels (Relative mRNA, arbitrary units (a. u.)) attained by any given Formin in the control knockdown cells were used as a reference. Data represent mean ± SD (n = 3, Two-way ANOVA). Note that DIAPH3 is the human homologue of mDia2. Delphilin expression is not depicted because we could not obtain single amplicons (not shown). FMNL3 is not reported because its mRNA turned out to be absent in U2OS cells, as determined by using the primers reported in the Supplemental Experimental Procedures (not shown). (**C**) Validation of shRNA-mediated knockdown of mDia2 and FBXO3 expression levels. U2OS cells were infected with mDia2-targenting (mDia2 #1 and #2) and FBXO3-targeting lentiviruses (FBXO3 #1 and #2). Control lentiviral particles were used to generate the control knockdown (shCtr) cells. Total RNA and RT-qPCR studies were performed as in **B**. Data are expressed as in as in **B** (n = 6, Fisher's exact *t*-test). Silencing of mDia2 and FBXO3 was also confirmed by immunoblotting (not shown) and the most effective targeting sequences were used for the experiments depicted in Figure 7b-f.



Supplemental Figure 4

Supplemental Figure 5

mDia2, FBXO3 and p53 localize in the nucleus. U2OS cells plated on gelatin-coated coverslips were treated with DMSO or Etoposide (20 μM) for three hours, fixed and stained with anti-mDia2 (mDia2, red), anti-p53 (p53, blue) and anti-HA ((HA) FBXO3, green) antibodies. Representative confocal central sections are shown. Red box marks the area of inset shown as merge. All channels were acquired sequentially. Scale bar, 10 μm.



Supplemental Figure 5