REPORT

Dysfunctional mitochondrial fission impairs cell reprogramming

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ABSTRACT

We have recently shown that mitochondrial fission is induced early in reprogramming in a Drp1-dependent manner; however, the identity of the factors controlling Drp1 recruitment to mitochondria was unexplored. To investigate this, we used a panel of RNAi targeting factors involved in the regulation of mitochondrial dynamics and we observed that MiD51, Gdap1 and, to a lesser extent, Mff were found to play key roles in this process. Cells derived from Gdap1-null mice were used to further explore the role of this factor in cell reprogramming. Microarray data revealed a prominent down-regulation of cell cycle pathways in Gdap1-null cells early in reprogramming and cell cycle profiling uncovered a G2/M growth arrest in Gdap1-null cells undergoing reprogramming. High-Content analysis showed that this growth arrest was DNA damage-independent. We propose that lack of efficient mitochondrial fission impairs cell reprogramming by interfering with cell cycle progression in a DNA damage-independent manner.

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Introduction

Mitochondria join by fusion and divide by fission. The balance between mitochondrial fission and fusion determines the shape and function of these organelles. Given the importance of mitochondria for cell homeostasis, these 2 processes play an active role in the regulation of cell growth, survival and cell death. Importantly, mutations in genes encoding for proteins controlling mitochondrial fusion and fission have been linked to a wide range of human diseases, underscoring the importance of understanding the regulation of mitochondrial dynamics in different biological processes.^{1–6}

Mitofusin-1 and -2 (Mfn1 and Mfn2) and Optic atrophy 1 (Opa1) proteins located in the outer and inner mitochondrial membranes, respectively, are known to mediate the fusion of this organelle. The Dynamin related protein-1, Drp1 is a key factor which mediates mitochondrial fission.^{7–11} Mitochondrial fission starts with the formation of an initial constriction in the mitochondria at contact sites with the endoplasmic reticulum.^{12,13} Activated Drp1 is then recruited to the constricted mitochondrial membrane, forming a ring that eventually fragments mitochondria in a GTPase-dependent manner.^{2,6} Recruitment of activated Drp1 to the mitochondrial surface is mediated by accessory proteins located at the external membrane of these organelles and include Mitochondrial fission factor, Mff,^{14,15} Mitochondrial fission protein 1, Fis1^{15,16} and Mitochondrial elongation factor 1 and 2, Mief1/MiD45 and

Mief2/MiD51,^{15,17,18} respectively, though the exact details of the interplay between activated Drp1 and the different factors on the mitochondrial surface are not completely known. The *GDAP1* gene (MIM #606598) is linked to Charcot-Marie-Tooth disease¹⁹ and has been shown to play a role in regulating mitochondrial dynamics in human.²⁰ Although the mechanisms whereby *GDAP1* participates in the regulation of mitochondrial dynamics are not fully understood, its overexpression led to the fragmentation of the mitochondrial network in human cells whereas knockdown of *GDAP1* enhanced the tubular aspect of these organelles in mammalian^{21–23} and insect²⁴ cells.

Somatic cells from mouse or human origin can be reprogrammed to induced-Pluripotent Stem (iPS) cells by forced expression of *Oct4* (also known as *Pou5f1*), *Klf4*, *Sox2* and *cMyc*^{25–27} (named as OSKM herein). Our previous work has uncovered the importance of mitochondrial fission controlled by a Drp1-ERK axis in cell reprogramming.²⁷ Here, we have investigated the importance of the factors involved in Drp1 recruitment to mitochondria in OSKM-induced cell reprogramming using an RNAi approach and found that MiD51 and Gdap1 were the factors whose reduction affected both processes the most. By exploring in detail the role of Gdap1 in cell reprogramming, we found that dysfunctional mitochondrial fission caused by lack of this protein impairs cell reprogramming by interfering with cell cycle progression in a DNA damage-independent manner.

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Results

Identification of factors involved in OSKM-induced mitochondrial fission

We have observed that OSKM-mediated cell reprogramming induces mitochondrial fission early during the process.²⁸ To investigate the factors involved in the regulation of mitochondrial fission during cell reprogramming, we conducted a reprogramming assay where we knocked down factors known to play a major role in controlling mitochondrial fission by RNA interference.

Early-passage wild type Mouse Embryonic Fibroblasts (MEFs) were transduced with OSKM-encoding retroviruses. Next day, infected cells were transfected with different endoribonuclease-prepared siRNAs (esiRNAs) targeting *enhanced Green Fluorescent Protein* (eGFP), as control, or the indicated pro-fission factors. Twenty-five days after retroviral delivery of OSKM in the presence of the indicated esiRNAs, we estimated reprogramming efficiency in the cultures using an Alkaline Phosphatase (AP) assay (Fig. 1A). Compared with the control, knockdown of *MiD49* or *Fis1* did not have any effect in the emergence of AP-positive colonies. Knockdown of *Mff* showed a mild reduction in the emergence of AP-positive colonies relative to the control.

Interestingly, a reduction of *MiD51* or *Gdap1* mRNA levels led to a profound decrease in the numbers of AP-positive colonies when compared to control esiRNA. All the transfected esiRNAs reduced the expression levels of the targeted factors by at least 75% (Supplementary Fig. 1A), as assessed by quantitative Polymerase Chain Reaction (qPCR) 6 days after OSKM-transduction.

We next examined the effect of knocking down the different factors known to play a role in regulating mitochondrial dynamics in OSKM-induced mitochondrial fission. For this we measured mitochondrial morphology in OSKM-infected MEFs transfected with the indicated esiRNA constructs 6 days after viral transduction by immunofluorescence (IF) staining for the mitochondrial marker Tom20 (Fig. 1B). Six days after OSKM-expression, $31.8 \pm 1.5\%$ of cells transfected with the control esiRNA displayed fragmented mitochondrial morphology (Fig. 1B). Compared to the control, knockdown of *Fis1*, *Mff* or *MiD49* did not have an effect in the mitochondrial morphology of OSKM-infected cells (Fig. 1B), suggesting that these proteins do not play an active role in OSKM-induced mitochondrial fission during early reprogramming. Remarkably, and relative to the control, reduction of *MiD51* or *Gdap1* mRNAs decreased OSKM-induced mitochondrial fragmentation by more than 50% (Fig. 1B). Overall, our findings suggest

Figure 1. Knockdown of pro-fission factors impairs mitochondrial fission and cell reprogramming. (A) Graph showing the number of Alkaline Phosphatase (AP)-positive colonies obtained in wild type MEFs transfected with the indicated esiRNAs after 25 days of retroviral delivery of the OSKM factors, ($n = 3$; $^{**}P < 0.01$; $^{****}P < 0.0001$). Panels in the right show representative bright field images and a magnification (inset) of the plates of the indicated cultures after AP-staining. (B) Left panels, representative confocal images of OSKM-transduced cells transfected with the indicated esiRNAs that were stained with anti-Tom20 antibody (red). Inset shows a magnification of the indicated area in the images of the left. Hoechst (blue) was used as a nuclear counterstaining. Scale bar $24 \mu\text{m}$. Graph on the right shows the quantification of the different mitochondrial morphologies observed in the cells treated as before, ($n = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). Data are represented as mean $\pm$ s.e.m. One-tailed unpaired Student's t-test was used to compare data sets.

that OSKM-mediated mitochondrial fission and cell reprogramming depends on the presence of MiD51, Gdap1 and, to a lesser extent, of Mff. These results prompted us to investigate further the role of Gdap1 in the reprogramming process.

Lack of Gdap1 impairs cell reprogramming and OSKM-induced mitochondrial fission

The above results suggested that lack of Gdap1-dependent mitochondrial fission could impair cell reprogramming. To investigate this possibility further, we carried out reprogramming assays with MEFs isolated from *Gdap1* knockout mice.²⁹ Interestingly, we observed a reduction of approximately 75% in the number of AP-positive colonies in Gdap1-null cells subjected to reprogramming when compared to wild type controls (Fig. 2A). No defects in cell proliferation under normal cell growth conditions or viral transduction efficiency were found in Gdap1-null cells when compared to wild type controls (Fig. S1B, C). These results support the notion that lack of Gdap1 impairs cell reprogramming. Nonetheless and albeit with a much lower efficiency than with wild type cells, we were able to isolate iPS-like colonies derived from Gdap1-null MEFs. Molecular and functional analysis showed that the isolated Gdap1-null cell clones were *bona fide* iPS cells (Figs. S2 and S3).

We monitored mitochondrial dynamics in MEFs by IF as above. In agreement with its proposed role in the fission process and the results shown above, at day zero the majority of Gdap1-null cells were classified as tubular (Fig. 2B, right panels and graph). Compared to wild type controls, Gdap1-null MEFs showed a decreased induction of mitochondrial fragmentation during reprogramming and only $22.44 \pm 3.80\%$ of the cells showed fragmented mitochondria 4 days after OSKM expression *versus* $35 \pm 0.5\%$ found in wild type counterparts (Fig. 2B, graph). These results indicate that lack of *Gdap1* interferes with OSKM-induced mitochondrial fragmentation as well as with the fission of these organelles in normal settings.

During reprogramming, cells undergo Mesenchymal-to-Epithelial Transition (MET).^{30,31} In wild type cells, Phalloidin and Tom20 staining of the cultures at day 8 of reprogramming showed mesenchymal cells displaying tubular organization of mitochondria whereas epithelial-like colonies showed a fragmented arrangement of these organelles (Fig. 2C). Interestingly and similar to wild type controls, 85% of the cells in Gdap1-null colonies displayed also fragmented mitochondria (Fig. 2, middle panels, inset and graph). These results support the notion that Gdap1 does not play a role in the regulation of mitochondrial dynamics once cells undergoing reprogramming have achieved the epithelial state to continue their path to pluripotency. In fact, lack of Gdap1 impaired the formation of epithelial-like colonies when compared to wild type MEFs (Fig. 2D). In keeping with this, qPCR analysis of *Gdap1* expression showed that the mRNA levels of this gene decreased at day 4 of reprogramming and were completely absent in pluripotent cells (Fig. 2E). Altogether, these results indicate that Gdap1-dependent mitochondrial fission in MEFs is important for initiating the transit to pluripotency before reaching, or at, the MET barrier to pluripotency.

Lack of Gdap1 does not alter the expression of mitochondrial dynamics regulatory factors

We next sought to investigate whether the observed decrease in OSKM-induced mitochondrial fission associated with the lack of *Gdap1* gene was due to an alteration in the expression and/or activation of known mitochondrial dynamics regulatory factors. For this, we first examined the expression of these factors in MEFs, OSKM-expressing cells and pluripotent cells from both wild type and *Gdap1*-null genotypes, using qPCR (Fig. 3A). As we described previously,²⁸ the expression of the majority of the factors involved in mitochondrial dynamics increased during reprogramming. Interestingly, cells from both genotypes displayed the same profiles of gene (Fig. 3A) and protein (Fig. 3B, C) expression during cell reprogramming and in iPS cells. In addition, we did not find significant differences between cells of both genotypes in the phosphorylation of S579-Drp1, an indication of Drp1 activation,⁷⁻¹¹ neither in response to OSKM expression (Fig. 3D) nor in self-renewing pluripotent stem cells (Fig. 3E). These results indicate that lack of *Gdap1* gene did not alter the expression nor activation of the protein set that regulates mitochondrial dynamics neither in cells undergoing cell reprogramming nor in pluripotent stem cells.

Lack of Gdap1 induces a DNA damage-independent G2/M cell arrest early during reprogramming

We next examined the transcriptional response to OSKM-expression in wild type and Gdap1-null cells using microarrays. Comparative gene expression analysis identified 5,802 differentially expressed genes (DEGs) at day 4 of reprogramming between wild type and Gdap1-null cells (Fig. 4A and Table S1). Gene Ontology analysis revealed that genes related to cell division, protein catabolic processes or RNA processing/modification were highly downregulated in Gdap1-null cells compared to wild type controls (Fig. 4B). Furthermore, functional analysis of microarray data using Gene Map Annotator and Pathway Profiler (GenMAPP) revealed a prominent downregulation of cell cycle pathways in Gdap1-null cells at day 4 of reprogramming (Fig. 4B and Table S1). The results shown above suggested that cell cycle progression was compromised in OSKM-transduced *Gdap1* knockout cells. To validate the results from the microarray analysis on cell cycle, we carried out cell cycle profiling of the cells at day 4 of reprogramming by flow cytometry. No significant differences in the cell cycle profiles between wild type and Gdap1-null cells were found before transduction with OSKM-encoding retroviruses (Fig. 4C). Interestingly, we observed a statistically significant 3-fold increase in the proportion of OSKM-expressing Gdap1-null cells in G2/M when compared with wild type counterparts (Fig. 4C), confirming the results from the functional analysis of microarray data.

The initial regulatory network induced by OSKM expression promotes both reprogramming and apoptosis.³² Although our microarray data showed that Gdap1-null and wild type cells responded differently to OSKM-dependent transcription (Fig. S4A and Table S1), we did not find differences in the induction of apoptosis between both genotypes at day 4 of reprogramming, as measured by AnnexinV-staining using flow

Figure 3. Expression of mitochondrial dynamics regulatory factors in somatic and pluripotent cells. (A) Total RNA was extracted from wild type (black bars) or Gdap1-null (red bars) MEFs left untreated (control) or OSKM-infected for 4 days (OSKM), or from the indicated pluripotent cells (iPSCs). The expression of the indicated genes was then assessed by qPCR and represented as relative gene expression normalized to control wild type MEFs of the corresponding genotype. Gene expression of the indicated genes in E14Tg2a ES cells (ESCs) is included as control (white bars), ($n = 3$; $**P < 0.01$; $***P < 0.001$). (B-E) Pluripotent cells or MEFs of the indicated genotypes, were left untreated (control) or OSKM-infected (OSKM) for the indicated days. Then, cellular lysates were analyzed by immunoblotting using anti-Opa1 (B), anti-Mfn2 (C), or anti-phospho S579S-Drp1 or anti-Drp1 antibodies (D, E), as indicated. Tubulin antibody, shown in the lower panels, was used as a loading control.

Discussion

Mitochondrial fission controlled by a Drp1-ERK axis constitutes an early and necessary step in OSKM-induced reprogramming.^{28,34} Here, we identified MiD51, Mff and Gdap1 as key factors in mediating the remodelling of mitochondria early in reprogramming.

The role of MiD51 in mitochondrial dynamics regulation is controversial,¹⁸ as knockdown of the gene encoding this protein in different labs induced either mitochondrial fragmentation¹⁵ or mitochondrial fusion.¹⁶ Our results showing that

knockdown of *MiD51* induced mitochondrial elongation in MEFs support a role for this protein as a pro-fission factor. In addition, it has been suggested that MiD51 and Mff are part of a common Drp1-dependent regulatory pathway to control mitochondrial morphology³⁵ and MFF overexpression induced mitochondrial fission in immortalized fibroblasts.³⁶ As *Mff* knockdown had little effect in cell reprogramming in our system, we postulate that MiD51 could function independently of Mff to mediate mitochondrial fragmentation in response to OSKM expression. Interestingly, knockdown of *Gdap1* impaired mitochondrial fission in response to OSKM

Figure 4. Molecular analysis of cell reprogramming in the absence of Gdap1. (A) Venn diagram showing overlap of Differentially Expressed Genes (DEGs) among control and OSKM-transduced MEFs data sets from both genotypes. (B) GenMAPP analysis comparing OSKM-transduced wild type and Gdap1-null data sets. All downregulated (top, negative Log of ratio) or upregulated (bottom, positive Log of ratio) pathways in Gdap1-null cells relative to wild type MEFs at day 4 of reprogramming are shown. (C) Graphs showing the assessment of the percentages of cells in the different phases of the cell cycle at day zero (left bars diagram) or day 4 of reprogramming (right bars diagram), ($n = 3$; $**P < 0.01$). Histogram on the right shows the cell cycle distribution of the indicated genotypes at day 4 of reprogramming. (D) Representative dot plots of wild type or Gdap1-null MEFs transduced with the OSKM factors that were stained with Propidium Iodide (PtdIns) and FITC-conjugated Annexin V for assessing cell death 4 days after viral transduction. Data in the plots are represented as mean $\pm$ s.e.m. ($n = 3$, differences were non significant). (E) Graph showing the quantification of the data corresponding to wild type or Gdap1-null MEFs transduced with OSKM viruses for 4 days, processed for immunofluorescence with anti- γ H2AX- or -p53BP1 antibodies and analyzed by High-Content microscopy ($n = 3$, differences were non-significant). Data are represented as mean $\pm$ s.e.m. One-tailed unpaired Student's t-test was used to compare data sets.

expression at similar levels than *MiD51*. It is therefore tempting to suggest that, similar to the role performed by MFF in cancer cells, Gdap1 cooperates with *MiD51* to drive mitochondrial

fission in response to reprogramming stimuli, using a specific Drp1-dependent pathway. In addition, the formation of mitochondria-Endoplasmic Reticulum (ER) contacts plays a key

role in mitochondrial fission^{12,13} and Gdap1 has been shown to participate in the establishment of these contacts in neuroblastoma cells.²³ Thus, it is possible that lack of *Gdap1* impairs OSKM-induced mitochondrial fission by interfering with the formation of mitochondria-ER contacts, leading to a decrease in reprogramming efficiency. Interestingly, Gdap1 is absent in pluripotent cells whereas it participates in mediating mitochondrial fission in MEFs before and during the very early stages of reprogramming. Thus, cells undergoing reprogramming could use a similar set of proteins to that found in basal conditions while the regulation of mitochondrial morphology in the intermediate states of reprogramming and in pluripotent cells may employ different mechanisms and/or molecular players.

Cancer cells are rapidly cycling cells, as are cells undergoing reprogramming, which constitute a privileged subset of cells with an ultrafast cell cycle.³⁷ Interestingly, depletion of *FIS1* in human cancer cells caused elongation of mitochondria and DNA damage, followed by G2/M growth arrest induction to eventually trigger senescence.^{33,38,39} Although knockdown of *Fis1* showed that this protein does not participate in regulating mitochondrial morphology in our experimental system, reduction of *Gdap1*, *Mff* or *MiD51* mRNA levels in MEFs caused a similar elongation of mitochondria to that found upon *FIS1* knockdown in transformed cells. Interestingly, *Gdap1* knockout cells displayed a G2/M growth arrest early during reprogramming but conversely to that found with *FIS1* ablation in cancer cells, this growth arrest was not associated with DNA damage. Thus, it is possible that OSKM-expressing cells lacking *Gdap1* were forced to engage into rapid cell divisions without properly fragmenting mitochondria, leading to a G2 and/or M cell cycle checkpoint activation to halt proliferation. Remarkably, this growth arrest was DNA damage-independent and therefore, different to that observed in cancer cells upon *FIS1* knockdown. This suggests that cancer cells and cells undergoing reprogramming respond differently to the presence of abnormally elongated mitochondria in G2/M cell cycle phases. Finally, and although we were able to derive *bona fide* Gdap1-null iPS cells, interference with mitochondrial fission by knocking down *DRP1* caused aneuploidy in cancer cells.⁴⁰ Thus, our results also suggest that caution must be taken when deriving human iPS cells for modeling human diseases characterized by a dysfunctional mitochondrial dynamics, such as Charcot-Marie-Tooth, as their reprogramming in the absence of a proficient mitochondrial fission machinery could either be highly inefficient and/or render cytogenetically abnormal iPS cells.

Mitochondria and peroxisomes cooperate at the functional and structural levels.⁴¹ Functionally, these organelles cooperate in the regulation of the lipid and Reactive Oxygen Species homeostasis. At the structural level, mitochondria and peroxisomes share both molecular mechanisms and regulatory proteins for undergoing division. In fact, *Fis1*, *Mff* and *Gdap1* proteins are also present on peroxisomes and regulate the fission of these organelles in insect and mammalian cells.^{13,20} Due to the existing interplay between mitochondria and peroxisomes, the possible implication of a peroxisomal dysfunction, caused by the knockdown of *Mff*, *MiD51* or *Gdap1*, in negatively influencing cell reprogramming in our settings cannot be ruled out. This is, nonetheless, an interesting question that warrants further investigation in the future.

As iPS cells are important tools for disease modeling^{42,43} and defects in mitochondrial homeostasis are associated with human pathologies,⁵ our study has a broad interest not only for

iPS cells-derived biomedical applications but also for understanding the role of factors that participate in the regulation of mitochondrial dynamics in physiological as well as pathological settings.

Materials and methods

Cell culture, reprogramming assays, reagents and vectors

ES cells were cultured on gelatinised plates in ES cell medium supplemented with 10% Foetal Bovine Serum (FBS) (Cat. S182B, Biowest Europe, Nuaille, France) in the presence of LIF.⁴⁴ When indicated, ES and iPSCs were grown on gelatinised plates in 2i medium⁴⁵ [(1:1) mixture of Neurobasal:DMEMF12 (Cat. 11570556, Thermo Fisher Scientific, Madrid, Spain; and Cat. L0091, Biowest Europe, Nuaille, France, respectively) supplemented with $0.5 \times N2$, $0.5 \times B27$ (Cat. 11520536 and Cat. 11530536 both from Thermo Fisher Scientific, Madrid, Spain), $3 \mu M$ CHIR99021 and $1 \mu M$ PD0325901 (Cat. 361571 and Cat. 444968, both from Millipore, Madrid, Spain)] in the presence of LIF. PlatE⁴⁶ and SNL⁴⁷ cells were grown in DMEM containing 10% FBS. When indicated, SNL cells were mitotically inactivated by treatment with $10 \mu g ml^{-1}$ Mitomycin-C (Cat.

M4287, Sigma-Aldrich Quimica SL, Madrid, Spain) for 3 h at 37°C. Wild type and Gdap1-null MEFs (both in a pure C57BL/6 background) were prepared from pooled E13.5 embryos and cultured in DMEM supplemented with 10% FBS and Penicillin/Streptomycin. Early-passage MEFs (third passage at most) were used in all experiments. All cells have been routinely tested for mycoplasma contamination using the Lookout Mycoplasma PCR detection kit (Cat. MP0035, Sigma-Aldrich Quimica SL, Madrid, Spain). *Gdap1*^{-/-} mice²⁹ have been published elsewhere. The retroviral vectors pMX-Oct4, pMX-Sox2, pMX-Klf4 and pMX-c-Myc²⁵ were from Addgene. Mission esiRNA targeting *eGFP*, or mouse *Fis1*, *Mff*, *MiD49*, *MiD51* or *Gdap1* (Cat. EHUEGFP, EMU016691, EMU025311, ESIOPEN NM_001009927.2 COMPASS#AXM15-18183970, EMU006961 and EMU001231, Sigma-Aldrich Quimica SL, Madrid, Spain) were delivered into MEFs using Lipofectamine RNAiMAX (Cat. 10514953, Thermo Fisher Scientific, Madrid, Spain), as indicated.

Ecotropic retroviruses were produced in PlatE cells transfected using Polyethylenimine (PEI) "Max" (Mw 40,000) (Cat. 24765-2, Polysciences Europe GmbH, Hirschberg an der Bergstrasse, Germany) exactly as described.²⁵ For reprogramming wild type or Gdap1-null cells, 8×10^5 MEFs were plated per p100 mm the day before the assay. Next day (day 0), MEFs were incubated overnight with a 1:1:1:1 mixture of mouse *Oct4*, *Sox2*, *Klf4* and *c-Myc* retroviral supernatants supplemented with $4 \mu g ml^{-1}$ Polybrene. Next day the supernatants were replaced with fresh media and cells were incubated for 3 more days (day 4). For reprogramming in the presence of esiRNAs, 30×10^3 MEFs in p24 well plates were transduced as before with OSKM. Infected MEFs were then transfected overnight with $0.6 \mu g$ of the indicated esiRNAs at day 1 and day 3 post-infection, using Lipofectamine RNAiMAX. Next morning, media was replaced with fresh media. Six days after OSKM-infection, cells were used for either cell reprogramming assays, RNA extraction or immunofluorescence analysis.

For assessing reprogramming efficiency, 1.5×10^5 cells were plated on a confluent layer of mitotically-inactivated SNL

feeders seeded the day before on gelatine-coated p100 mm dishes at 2×10^6 cells per dish. Next day, media was changed to ES cell growth medium containing 10% FBS and LIF. Media was changed every other day. Reprogramming efficiency was assessed 25 days after transduction of MEFs with OSKM-encoding retroviruses by scoring all the alkaline phosphatase positive colonies per p100 mm. Alkaline phosphatase staining was performed using the Alkaline Phosphatase Detection kit (Cat. SCR004, Millipore, Madrid, Spain).

For iPS cell generation, colonies were hand-picked at day 25 of reprogramming, transferred to a 1.5 ml tube containing 50 μ l of Trypsin/EDTA solution and incubated for 10 minutes at 37°C. Then, cells were disaggregated with a pipette by adding 200 μ l of ES cell media containing 10% FBS and LIF. Cells were then plated on SNL feeders in p24 plates (1 clone per well) in ES cell media supplemented with 10% FBS and LIF. When colonies were macroscopically visible, media was switched to 2i medium with LIF. Surviving clones were further expanded in 2i media with LIF and analyzed as indicated.

Immunofluorescence and flow cytometry

For immunofluorescence analysis, all cells were plated on gelatine-coated coverslips. iPS and ES cells were seeded onto Mitomycin C-treated SNL cells and grown until clones were macroscopically visible. MEFs, untreated, transfected and/or transduced with the indicated viruses, were plated at 15000 cells per cm^2 the day before processing. Then, cells were fixed for 15 min at room temperature with 4% paraformaldehyde in PBS, permeabilised for 10 minutes with 0.5% Triton-X-100 in PBS, blocked for 30 minutes with blocking buffer [3% Bovine Serum Albumin (BSA, Cat. A7030, Sigma-Aldrich Quimica SL, Madrid, Spain) in PBS containing 0.025% Tween-20] and incubated overnight with primary antibodies in blocking buffer. After washing with PBS supplemented with 0.025% Tween-20, cells were incubated for 1 hour with the appropriate secondary antibodies in blocking buffer containing 5 μ g per ml of Hoechst 33258 (Cat. 11594876, Thermo Fisher Scientific, Madrid, Spain), washed overnight with PBS, mounted and analyzed using confocal microscopy. For 2-bromo-5-deoxyuridine (BrdU) staining, cells were treated before blocking with 2N HCl at 37°C for 15 min and then with 0.1 M borate buffer (pH 8.5) at room temperature for 10 min, followed by blocking and incubation with mouse anti-BrdU antibody (Cat. MAB4072, Sigma-Aldrich Quimica SL, Madrid, Spain Sigma-Aldrich). Colocalisation of Tom20 and GFP-LC3B or Tom20 and Drp1 stainings was evaluated by calculating the Pearson Correlation Coefficient (PCC) using the freely-available JACoP plug-in (<http://rsb.info.nih.gov/ij/plugins/track/jacop.html>) for ImageJ analysis software, as previously described.⁴⁸

Chromosome counting was carried out exactly as described⁴⁹ using Hoechst 33258 as DNA dye. At least 25 spreads per sample were analyzed.

Confocal immunofluorescence images were taken using a Fluoview FV10i (OLYMPUS IBERIA S.A.U., Hospitalet de Llobregat, Barcelona, Spain) confocal microscope equipped with 405-, 488- and 633-nm lasers. Three-dimensional reconstructions of z-stacks were performed using FV10-ASW 2.1 viewer software (OLYMPUS IBERIA S.A.U., Hospitalet de Llobregat, Barcelona, Spain). All images were further processed using

Adobe Photoshop CS5 and compiled using Adobe Illustrator CS5 (Adobe Systems Inc., San Jose, CA, USA). Alexa Fluor 488-Phalloidin was from Thermo Fisher Scientific (Cat. 10125092). Details about the antibodies used in this study are provided in Table S2.

For cell cycle profiling, trypsinised cells were fixed with ice-cold 70% ethanol for 30 minutes at 4°C, washed once with PBS and incubated in cell cycle propidium iodide/RNase solution (Cat. PI/RNASE, Immunostep, Salamanca, Spain) for 15 minutes in the dark at room temperature. For the analysis of apoptosis, MEFs were resuspended in AnnexinV-binding buffer, and stained with 634-conjugated AnnexinV/propidium iodide solution (Cat. ANNEXIN V DY634, Immunostep, Salamanca, Spain) for 15 minutes in the dark at room temperature. For mitochondrial mass assessment by flow cytometry, cells were trypsinised and resuspended in media containing 25 μ g per ml of the membrane potential-independent mitochondrial dye MitoTracker Green FM (Cat. 11589106, Thermo Fisher Scientific, Madrid, Spain), incubated at room temperature for 10 minutes and then processed for flow cytometry. All measurements were taken using a BD FACSCanto II or FACS Verse (both from BD Biosciences, San Jose, CA, USA) flow cytometers and analyzed using FlowJo V8.1.1 (Tree Star Inc., Ashland, OR, USA) software. At least 10,000 events from each sample were recorded.

High-content analysis

For the High-Content Analysis (HCA), 30000 MEFs were plated in 24-well plates (Nunc) and stained by immunofluorescence with the indicated antibodies. One fluorescence image was collected for Hoechst 33258 (405/535 nm dichroic) and another for AlexaFluor-549 (535/620 nm dichroic) to detect the different markers with the InCell Analyzer 2000 automated epifluorescence microscope (GE Healthcare, Paterna, Valencia, Spain) at 40x magnification. HCA acquisition was performed using InCell investigator Software (GE Healthcare, Paterna, Valencia, Spain). For the analysis, cells and nuclei were defined using the Hoechst 33258 staining. The nuclei were segmented using top-hat segmentation defining a minimum nucleus area of 50 μm^2 . To define the cell segmentation a collar segmentation routine was used, specifying a ratio of 4 μm . To analyze the expression of γ H2AX, p53BP1 or BrdU by cell, the average intensity of pixels in the reference channel (AlexaFluor-549) within the defined nuclear region was measured. Once each cell had associated a nuclear intensity for the specific expression, a threshold filter defining positive and negative expressing cells was set. Threshold filter uses a histogram for data visualization. To specify the filter settings, the nuclear intensity measure was selected. The threshold filter defines the cells with nuclear intensities above or below a given value as positive or negative respectively for the expression of the protein. As a result, the program assigns to each cell the definition of positive or negative for the expression of γ H2AX, p53BP1 or BrdU and generates a percentage of both cell populations (positive and negative) per well. Details about the antibodies used in this study are provided in Supplementary Table 2.

Western blot

Cells were lysed in lysis buffer [50mM Tris pH7.5, 150mM NaCl, 5mM EDTA, 0.5% Nonidet P-40, 100 mM NaF, 2 mM Na_3VO_4 ,

20 mM $\text{Na}_4\text{P}_2\text{O}_7$, and 1x complete proteinase inhibitor cocktail (Roche)]. Cellular lysates were used for immunoblotting with the indicated antibodies using standard procedures. Signals in western blots were detected using ECL prime (Cat. RPN2232, GE Healthcare, Paterna, Valencia, Spain) and images automatically captured in an ImageQuant LAS 4000 digital imaging system equipped with FUJINON F0.85 lenses and a Fujifilm super CCD area type chip (GE Healthcare, Paterna, Valencia, Spain). Acquired images were processed using ImageQuant TL 7.0 analysis software (GE Healthcare, Paterna, Valencia, Spain). Details about the antibodies used in this study are provided in Supplementary Table 2.

Nucleic acid isolation and qPCR analysis

Total RNA was extracted using TRI reagent (Cat. T9424, Sigma-Aldrich Quimica SL, Madrid, Spain) and cDNA synthesized using SuperScript III reverse transcriptase kit (Cat. 10432122, Thermo Fisher Scientific, Madrid, Spain). cDNA products were amplified using a StepOne Fast Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific, Madrid, Spain). Where indicated, Taqman probes were from Applied Biosystems. Sequences of the primers used in this study are listed in Supplementary Table 3.

Microarray data analyses

Microarray data (Affymetrix GeneChip Mouse Expression Array 430 2.0, Affymetrix) was standardized using Robust Multi-array Average method and quantile normalization. Differential gene expression was carried out using the *limma*⁵⁰ and *masigpro* packages from Bioconductor. Multiple testing adjustments of p-values were done according to Benjamini and Hochberg methodology. Gene set analysis was carried out for the Gene Ontology (GO) terms and for the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways using a logistic regression model,⁵¹ which returns adjusted p-values based on False Discovery Rate method. GO annotation for the genes in the microarray were taken from Ensembl 73 release. Microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO Series GSE59527).

Statistical methods

One-tailed Student's t-test was used to estimate statistical significance between categories. Relative values (percentages) were normalized using arcsine transformation before carrying out their statistical comparison. Results are presented as mean $\pm$ standard error of the mean (s.e.m.).

Ethics statement

All experiments were carried out in accordance with the guidelines of the Ethics committee at the University of Valencia. Mice were crossed and maintained at the University of Valencia animal core facility in accordance with Spanish regulations (RD53/2013). The experimental protocol (no. 2015/VSC/PEA/00079) was approved by the Animal Experimentation Ethics Committee of the University of Valencia and the Generalitat Valenciana government (Spain).

Abbreviations

AP	Alkaline Phosphatase
DEGs	differentially expressed genes
eGFP	enhanced Green Fluorescent Protein
ER	Endoplasmic Reticulum
esiRNA	endoribonuclease-prepared siRNAs
GenMAPP	Gene Map Annotator and Pathway Profiler
IF	Immunofluorescence
iPS cells	induced-Pluripotent Stem cells
MEF	Mouse Embryonic Fibroblast
MET	Mesenchymal-to-Epithelial Transition
OSKM	Oct4+Sox2+Klf4+cMyc
qPCR	quantitative Polymerase Chain Reaction

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

JP and ML conducted the majority of the experiments. XP, CL-G and RB helped with the microscopy analysis. MB-M and FP generated the Gdapl⁻ null mice. ES obtained the microarray data. FG-G and JD carried out the microarray data analysis. JP, ML and JT conceived the project and designed the experiments. All authors discussed the results. FP and JT provided the funding.

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