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► To cite this version:

Romain da Costa, Steven de Almeida, Martin Chevarin, Smail Hadj-Rabia, Stéphanie Leclerc-Mercier, et al.. Neutralization of HSF1 in cells from PIK3CA-related overgrowth spectrum patients blocks abnormal proliferation. Biochemical and Biophysical Research Communications, 2020, 530, pp.520 - 526. <10.1016/j.bbrc.2020.04.146>. <hal-03491260>

HAL Id: hal-03491260

<https://hal.science/hal-03491260v1>

Submitted on 22 Aug 2022

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Neutralization of HSF1 in cells from *PIK3CA*-related overgrowth spectrum patients blocks abnormal proliferation

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ABSTRACT

PIK3CA-related overgrowth spectrum is caused by mosaicism mutations in the *PIK3CA* gene. These mutations, which are also observed in various types of cancer, lead to a constitutive activation of the PI3K/AKT/mTOR pathway, increasing cell proliferation. Heat shock transcription factor 1 (HSF1) is the major stress-responsive transcription factor. Recent findings indicate that AKT phosphorylates and activates HSF1 independently of heat-shock in breast cancer cells. Here, we aimed to investigate the role of HSF1 in *PIK3CA*-related overgrowth spectrum. We observed a higher rate of proliferation and increased phosphorylation of AKT and p70S6K in mutant fibroblasts than in control cells. We also found elevated phosphorylation and activation of HSF1, which is directly correlated to AKT activation. Specific AKT inhibitors inhibit HSF1 phosphorylation as well as HSF1-dependent gene transcription. Finally, we demonstrated that targeting HSF1 with specific inhibitors reduced the proliferation of mutant cells. As there is currently no curative treatment for *PIK3CA*-related overgrowth spectrum, our results identify HSF1 as a new potential therapeutic target.

Keywords : HSF1, PI3K, Segmental overgrowth spectrum

INTRODUCTION

Segmental overgrowth spectrum, which can affect various tissues, results in subcutaneous, lipomatous, muscular, visceral, skeletal, cerebral, vascular and/or cutaneous hyperplasia. Postzygotic activating mutations in the phosphoinositide-3-kinase (PI3K)/AKT/mTOR pathway are a major cause of segmental overgrowth: *AKT1* mutations in Proteus syndrome [1], *PIK3CA*, *AKT3* and *MTOR* mutations in hemimegalencephaly [2], *PIK3CA* mutations in Congenital Lipomatous Overgrowth, Vascular malformations, and Epidermal nevi (CLOVES) syndrome [3] and other related disorders known as *PIK3CA*-related overgrowth spectrum (PROS). Mutations in the *PIK3CA* gene, which encodes the PI3K p110 α catalytic subunit, are found in hot spots such as p.His1047Arg or p.His1047Leu [4]. These mutations, which result in constitutive PI3K/AKT pathway activation, have also been observed in ovarian, breast, colorectal and hepatocellular carcinomas [5-7]. The use of specific inhibitors to target PI3K or mTOR in PROS was recently found to lead to disease regression [8,9]. However, the downstream effectors of the PI3K and mTOR pathways specifically involved in the pathogenicity of PROS remain unknown. Their identification could lead to the development of alternative targeted therapies with fewer side effects.

Heat shock transcription factor 1 (HSF1) is the main stress-responsive transcription factor [10,11]. It is constitutively expressed in all tissues, but its activation is only observed in conditions of cellular stress. HSF1 is activated by trimerization and hyperphosphorylation. Originally, it was described to bind to heat-shock elements in the promoter region of genes in response to heat shock and to positively regulate their expression [12]. This induction results in molecular chaperones expression also known as heat shock proteins (HSPs), such as HSP70. The accumulation of HSPs with protein folding and anti-aggregation properties, results in cell protection. HSF1 is also involved in development and cellular differentiation,

by regulating non-heat shock genes [13-15] including an extensive set of genes related to various processes such as apoptosis, RNA splicing and ubiquitination. Cancer cells also have a strong need for chaperones such as HSPs because they have to constantly rewire their metabolism [16,17]. In this context, HSF1 is upregulated in numerous cancers and cancer-associated fibroblasts, leading to the expression of gene sets associated with tumor proliferation, resistance to apoptosis and angiogenesis [18,19]. HSF1 activation is therefore strongly associated with poor cancer prognosis and outcome [18] [19]. The mechanisms of HSF1 activation are poorly understood, but it was recently shown that AKT phosphorylates and activates HSF1 independently of heat-shock, leading to epithelial-mesenchymal transition in breast cancer cells [20,21].

Because patients with PROS harbor somatic activating mutations in the PI3K/AKT/mTOR pathway, we have started to investigate HSF1 involvement in the development of these diseases. HSF1 inhibitors have already been clinically evaluated, making the identification of HSF1 a therapeutic target of great interest.

Here, we show that HSF1 is over-activated *in vivo* and in skin fibroblasts *in vitro*. Furthermore, the functional inhibition of HSF1 in those cells leads to a reduced cell cycle and cell growth.

MATERIALS AND METHODS

Cell Culture: Fibroblasts from healthy donors and patients with PROS (Table 1) were obtained after consent for skin biopsy of one of the affected areas (patients A and B). Patient A was a 4-year-old boy with diffuse thoracic lipomas and digital overgrowth. Patient B was a 7-year-old boy with prenatal overgrowth, cutaneous capillary malformation of the philtrum, thoracic reticulate capillary malformation, postaxial polydactyly and syndactyly (II-III), cutis

laxa. Fibroblasts were cultured in DMEM High Glucose medium (HyClone Thermo Scientific, Waltham, MA, USA) supplemented with 10% Fetal Bovine Serum (FBS, Thermo Scientific) and 1% ZellShield (Minerva, Biovalley, France). Cells were cultured at 37°C, 5% CO₂.

Immunofluorescence : Paraffin sections from patients N and T were obtained from Hôpital Necker, Paris, after patients, with informed consent, had surgical removal of overgrowth. Patient N had *PIK3CA*-related tissue dysplasia in the femur. Patient T had *PIK3CA*-related intraosseus lipoma. After deparaffinization and rehydration, sections were incubated in 0.1M TRIS-HCl pH8.8 buffer for 20 min at 80°C, blocked for 30 min at room temperature (PBS, 5 mg/ml Glycine, 10% FCS, 0.3% triton), and stained with anti-HSF1 antibody (Cell Signaling) overnight at 4°C, in PBS 5% FCS 0.05% Triton. Sections were washed with PBS, incubated with secondary antibody anti-rabbit Alexa 488 (Thermofisher) for 1 hour at room temperature, washed with PBS and incubated with anti-Phospho-AKT antibody (Abcam, Cambridge, UK) overnight at 4°C, in PBS 5% FCS 0.05% Triton. After washing, slides were incubated with anti-rabbit Alexa 568 (Thermofisher) for 1 hour at room temperature and washed. The same protocol was used for HSF1 staining on patient fibroblasts after the cells were cultured on glass slide for 48h and fixed with PFA 4% for 20 min.

Cell proliferation assay: Two thousand cells were plated into 96-well plates in DMEM with 10% FBS, 1% ZellShield. After 4h, the culture medium was switched to DMEM supplemented with 5% FBS, 1% ZellShield and increasing doses of Kribb11 (MerckMillipore, Milton, UK), Triptolide (Merck Millipore), BYL719 (MedChemExpress, NJ, USA), 4 ng/ml rapamycin (Sigma-Aldrich, Saint Louis, MO, USA), 10 µM LY294002 (Sigma-Aldrich). After 48 hours of culture, cell growth and viability were determined by adding XTT solution (#11465015001) (Roche Molecular Biochemicals, Mannheim,

Germany) for 5 hours at 37°C. Absorbance was then read at 490 nm using a 96-multiwell microplate reader (Multiskan GO, Thermofisher).

BrdU staining: After 24 hours with or without drugs, cells were incubated for 2 hours with 10 µM BrdU. BrdU incorporation was then quantified using the colorimetric cell proliferation ELISA kit (#11647229001) (Roche Molecular Biochemicals, Mannheim, Germany).

Cell death detection ELISA: After 24 hours with or without drugs, cell death (apoptosis and necrosis) was quantified using the cell death detection ELISA kit (#11774425001) (Roche Molecular Biochemicals, Mannheim, Germany).

Western blot analysis: Cell lysates were run on 10% SDS-PAGE gel and transferred to an Immobilon-P membrane (MerckMillipore). The membrane was blotted with antibodies in PBS, 0.05% Tween 20, 5% non-fat dried milk, washed and probed with HRP conjugated-secondary antibodies (Santa Cruz Biotechnology Inc., CA, USA). Antibody binding was visualized using the Clarity™ Western ECL substrate (Bio-Rad Laboratories, Inc, CA, USA). The primary antibodies used were anti-phospho HSF1 from Abcam (#ab76076) (Cambridge, UK), anti-HSF1 (#4356), anti-phospho AKT (Ser⁴⁷³) (#4060) and anti-AKT (#9272) from Cell Signaling Technology (Danvers, MA, USA); anti-vinculin (#V9131) and anti-Actin (A3854) were from Sigma-Aldrich.

Bioplex array: Detection of AKT and p70S6Kinase phosphorylation was quantified using MILLIPLEX® map phospho-AKT (Ser⁴⁷³), AKT, phospho-p70S6Kinase (Thr⁴¹²), p70S6Kinase magnetic beads MAPmates (#46-602MAG) (MerckMillipore). Results were read using the Bio-Plex® 200 Systems (Bio-Rad Laboratories).

RNA extraction and real-time RT-qPCR: Total RNA was extracted using TRI reagent® (Sigma Aldrich). RNA concentration and purity were measured using the Multiskan GO from

ThermoFisher. First-strand cDNA was synthesized using the iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories). The real-time qPCR was performed with iTaq™ SYBR® Green supermix (Bio-Rad Laboratories). PCR was performed in triplicate in 96-well plates, using the CFX96™ (Bio-Rad Laboratories). The custom HSF1 signature PCR plate was from Bio-Rad Laboratories. Other primer sequences are available upon request.

RESULTS

The active form of HSF1 is present in *PIK3CA* mutated tissues and skin fibroblasts from patients with *PIK3CA*-related overgrowth spectrum

Tissues from *PIK3CA*-mutated patients (patients N and T) were stained for the presence of P-AKT and HSF1 by immunofluorescence. Those tissues are heterogeneous on a mutational status (Table 1) and we observed a marked expression of HSF1 in cells with high levels of P-AKT (full arrow), but not in cells without AKT activation (dashed arrow), suggesting an association between the PI3K/AKT pathway and the activation of HSF1 in those patients (Figure 1A). We then used skin fibroblast samples from two PROS patients to gain more insight (patient A and B). *PIK3CA* next generation sequencing on cultured skin fibroblasts from both patients identified a hotspot p.His1047Arg mutation in 49% of alleles in patient A (100% mutant cells), and the more rare p.Cys378Tyr mutation in 30% of alleles in patient B (60% mutant cells). Mutant cells in both patients had a higher proliferation rate than control cells (Figure 1B), and exhibited increased phosphorylation of AKT (Figure 1C). Known PI3K/AKT targets are also increased like the cell cycle regulator CCND2 (Figure 1C) and the phosphorylated p70S6K (Figure 1D). This is in keeping with findings of Parker *et al*,

who reported that endogenous *PIK3CA* mutations lead to the activation of the PI3K/AKT pathway in affected fibroblasts with increased proliferation [22].

Besides the stronger phosphorylation of AKT observed in patients A and B than in the two non-mutated control fibroblasts, we also observed a strong expression of the phosphorylated form of HSF1 in patients' fibroblasts (Figure 1E). As patient A cell culture was entirely composed of mutant cells, we chose to perform the following experiments with this cell culture only. As a transcription factor, active HSF1 must localize in the nucleus. We observed by immunofluorescence, that P-HSF1 was indeed present in the nucleus of cells from patient A, but only slightly in normal fibroblasts (Figure 1F). Accordingly, higher expression of HSPs was observed in patient A fibroblasts (Figure 1G).

HSF1 activation is reduced by inhibitors of the PI3K/AKT pathway

Next, we sought to establish whether the PI3K/AKT/mTOR pathway was directly responsible for the activation of HSF1 in the fibroblast samples. The addition of insulin to activate the PI3K/AKT pathway in normal fibroblasts has been thoroughly described [23]. We observed an induction of HSF1 phosphorylation in these conditions as well (Figure 2A), suggesting that HSF1 activation was not *PIK3CA* mutation-dependent, but rather a consequence of PI3K/AKT pathway hyperactivation. Furthermore, inhibition of PI3K or mTOR activity by LY294002 and Rapamycin, respectively, strongly reduced HSF1 phosphorylation in control and patient cells (Figure 2B). Accordingly, P-HSF1 was no longer localized in the nucleus of these cells (Figure 2C), suggesting that HSF1-dependent gene expression was neutralized as well. In order to test this hypothesis, upon PI3K inhibition we determined the mRNA expression of 74 genes that are known to be regulated by HSF1 and overexpressed by tumor cells or in the tumor microenvironment [18,19] (Figure 2D). In 2 independent experiments, 47 genes were repeatedly down-expressed and 11 were up-expressed. The results were variable

for 12 other genes. Therefore, the majority of a cancer-related signature dependent on HSF1 can be inhibited by PI3K inhibition, confirming the link between those two pathways.

Targeting of HSF1 by specific inhibitors reduces fibroblasts proliferation from patients with *PIK3CA*-related overgrowth spectrum

The incubation of patient fibroblasts with specific HSF1 inhibitors (the well-established Triptolide [24] and the more recently published Kribb11 [25] resulted in a reduction of cell growth and total cell number as potent as with PI3K or mTOR inhibitors (Figure 3A, B and C). A clinically tested inhibitor of the PI3K/AKT pathway (BYL719) has been shown to improve the disease symptoms of several PROS patients [8]. We observed that Kribb11 was more potent *in vitro* than BYL719 in inhibiting the fibroblasts growth from patient A. (Figure 3D). The increased number of cells entering the S phase of the cell cycle, which is characteristic of PROS [26] was strongly reduced by Kribb11 (Figure 3E). Accordingly, CCND2, a PI3K/AKT target that regulates cell cycle, was down-expressed upon inhibition of HSF1 by the use of either Kribb11 or Tritoplid.(Figure 3F). This inhibition of proliferation was not associated with an increase in cell death (Figure 3G).

DISCUSSION

Though they are normal physiological processes, cell differentiation and proliferation entail enormous amounts of gene expression rewiring and protein synthesis. These conditions are observed during embryogenesis but also throughout life, as illustrated by hematopoiesis. These biological processes must be tightly controlled to avoid the aberrant development that is seen in such diseases as PROS. Here, we investigated the expression and activation status of HSF1 in PROS and showed that this transcription factor is involved in the growth of

mutated cells. HSF1, though originally described as the master transcription factor for molecular chaperones induced upon heat-shock, has been implicated in a much wider spectrum of gene regulation and cell functions. Our previously published findings had revealed that HSF1 modulates Spi1/PU.1 expression in immune cells at the transcriptional and post-translational levels in human monocytes undergoing macrophage differentiation, thus finely tuning myeloid differentiation [15]. HSF1 is also essential for *Drosophila* development without affecting *HSP* gene expression [27,28]. In *Hsf1*^{-/-} mice, multiple phenotypes reflecting defects in development were found: chorioallantoic placenta defects, prenatal death, growth retardation, female infertility [29-33]. Furthermore, the JNK-dependent and HSP-independent involvement of HSF1 in the promotion of organ growth has recently been demonstrated in mice [34]. This function is probably organ-dependent seeing as the constitutive expression of an active form of HSF1 in mice resulted, surprisingly, in the blockade of spermatogenesis [35]. Despite the clear implication of HSF1 in these differentiation processes, the present study is the first to investigate and describe the aberrant activation of HSF1 in a developmental disease.

In breast cancer, colon cancer and lung carcinoma, HSF1 is a key promoter of tumor development, metastasis and death [18]. In tumor cells, HSF1 controls the transcription of an array of genes independent of HSPs but related to cell signaling, DNA repair, translation and protein folding. Furthermore, the HSF1-cancer signature is associated with poor patient outcomes. Non-tumor cells from the micro-environment also see increased HSF1 activation resulting in a specific transcriptional program that promotes nearby tumor growth [19]. We observed that many genes selected from both signatures were inhibited by PI3K pathway neutralization, suggesting that cancer cells, cells from cancerous micro-environments and segmental overgrowth syndrome cells share some HSF1-dependent growth mechanisms. The impact of HSF1 is probably not limited to this set of genes but rather involves many other

genes controlling metabolism, cytoskeleton remodeling and other processes. Further investigation is warranted to clarify their respective roles in the PI3K-dependent overgrowth syndrome.

Considering the general impact of HSF1 deficiency in mice and the increasing availability of next generation sequencing, it would be interesting to look for mutations in the *HSF1* gene or gene amplification in rare genetic syndromes affecting development. So far, no mutations have been described in the *HSF1* gene, and only one inactivating mutation has been shown to affect a HSP in cancer [36].

Though the mechanisms that drive HSF1 activation are poorly understood, it is known that transcriptional activity can be repressed through phosphorylation of the regulatory domain by ERK [37]. Conversely, MEK physically interacts with, phosphorylates and activates HSF1 at S326 [38]. It would therefore be of interest to investigate the involvement of HSF1 in RASopathies, a group of rare genetic conditions caused by mutations in genes from the RAS/MAPK pathway.

Inhibitors of mTOR and PI3K have been recently used in clinical trials of PROS [8,9]. Results have been either disappointing or spectacular, and side effects have been variously described. The reason for this discrepancy of outcomes could be that PI3K and mTOR are upstream signaling molecules which control a large number of downstream signaling effectors. In contrast, among this wide signaling network, HSF1 is a more downstream target and our data suggest that its over-activation might be responsible for the aberrant cell behavior observed in PROS. Therefore, we believe that HSF1 targeting would better prevent some side effects observed with PI3K/mTOR targeting while still inhibiting aberrant cells growth (patent WO201986922).

Therapeutic options for developmental diseases of genetic origin are almost nonexistent, but our increasing understanding of cell signaling pathways and protein interactions will hopefully soon result in new targeted therapies. We believe that HSF1 is a promising candidate that deserves more investigation in segmental overgrowth spectrum and other genetic disorders.

ACKNOWLEDGMENTS

We would first like to thank the patients and their families for agreeing to participate in this study. We also thank MCM.network, the Conseil Regional de Bourgogne, the Fonds européen de développement régional, and the Université of Bourgogne (Bonus Qualité Recherche 2016) for providing funding for this project. We thank the Biological Resource Center Ferdinand Cabanne (BB-0033-00044, Dijon, France)

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TABLE

Table 1: Genetic data for patient fibroblasts

| patient | Mutated gene | isoform | variation (hg19) | cDNA | AA | % of mosaicism level in fibroblasts culture |
|---------|---------------|-------------|---------------------|-----------|--------------|---|
| A | <i>PIK3CA</i> | NM_006218.2 | chr3:g.178952085A>G | c.3140A>G | p.His1047Arg | 49 |
| B | <i>PIK3CA</i> | NM_006218.2 | chr3:g.178922364G>A | c.1133G>A | p.Cys378Tyr | 30 |
| N | <i>PIK3CA</i> | NM_006218.2 | chr3:g.178952085A>T | c.3140A>T | p.His1047Leu | na |
| T | <i>PIK3CA</i> | NM_006218.2 | chr3:g.178952085A>T | c.3140A>T | p.His1047Leu | na |

na: not applicable

FIGURE LEGENDS

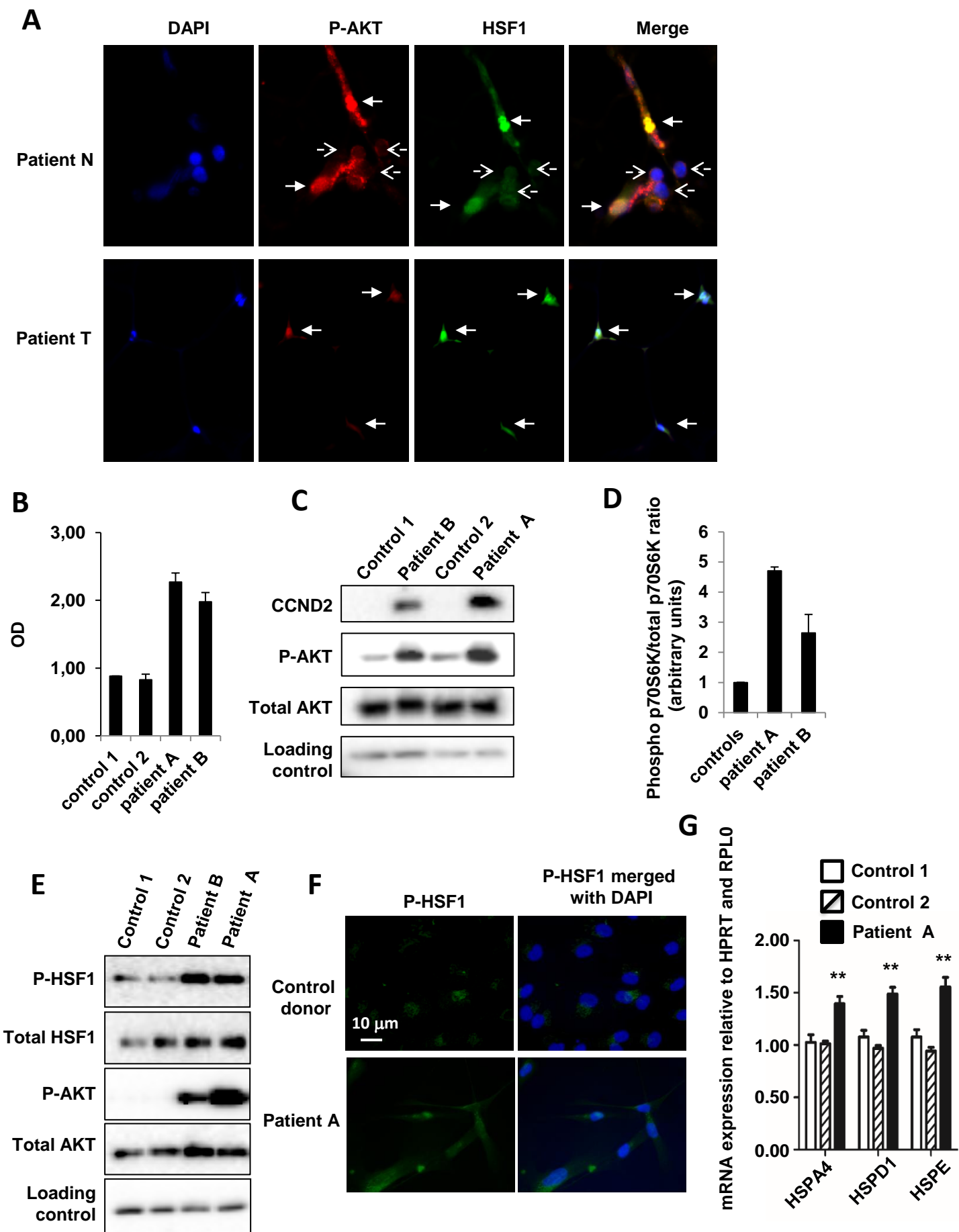
Figure 1: Increased HSF1 activity in *PIK3CA*-mutated patients. (A) Immunofluorescence on biopsies from two *PIK3CA*-mutated patients reveals correlation of HSF1 and P-AKT expression (full arrow indicates both strong P-AKT and HSF1 expression; dashed arrow indicates low expression of both). (B) Quantification of proliferation by XTT staining for

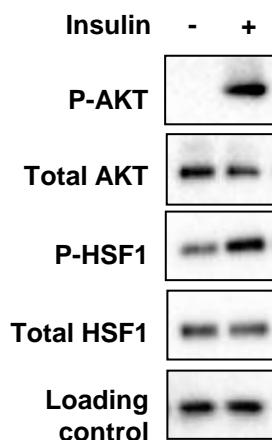
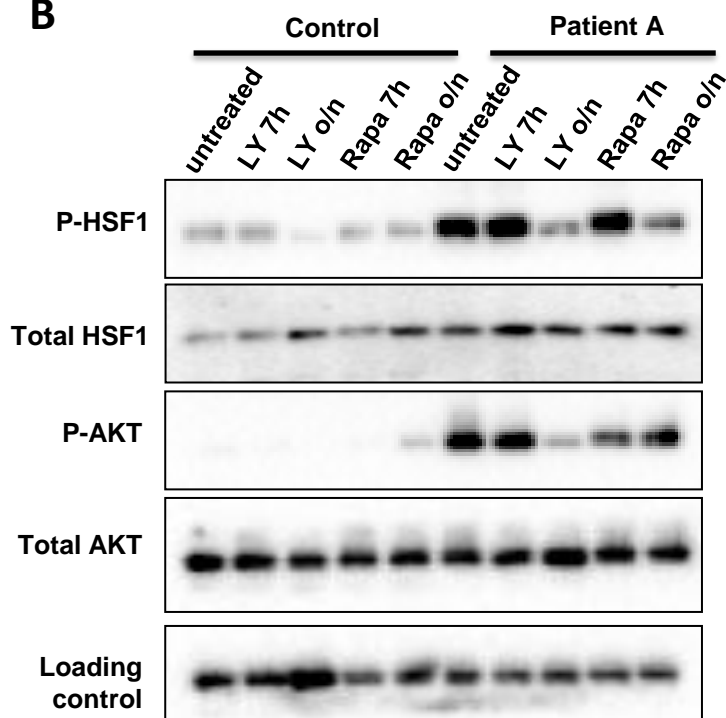
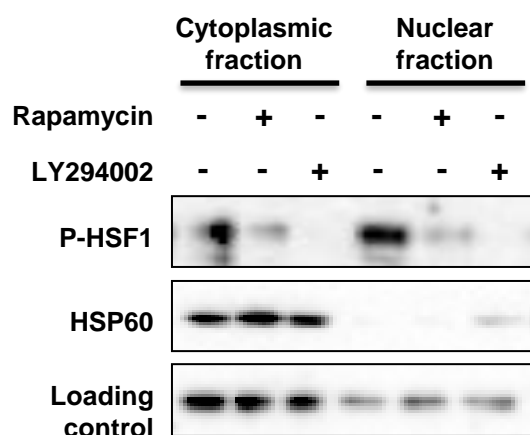
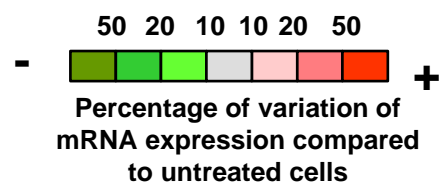
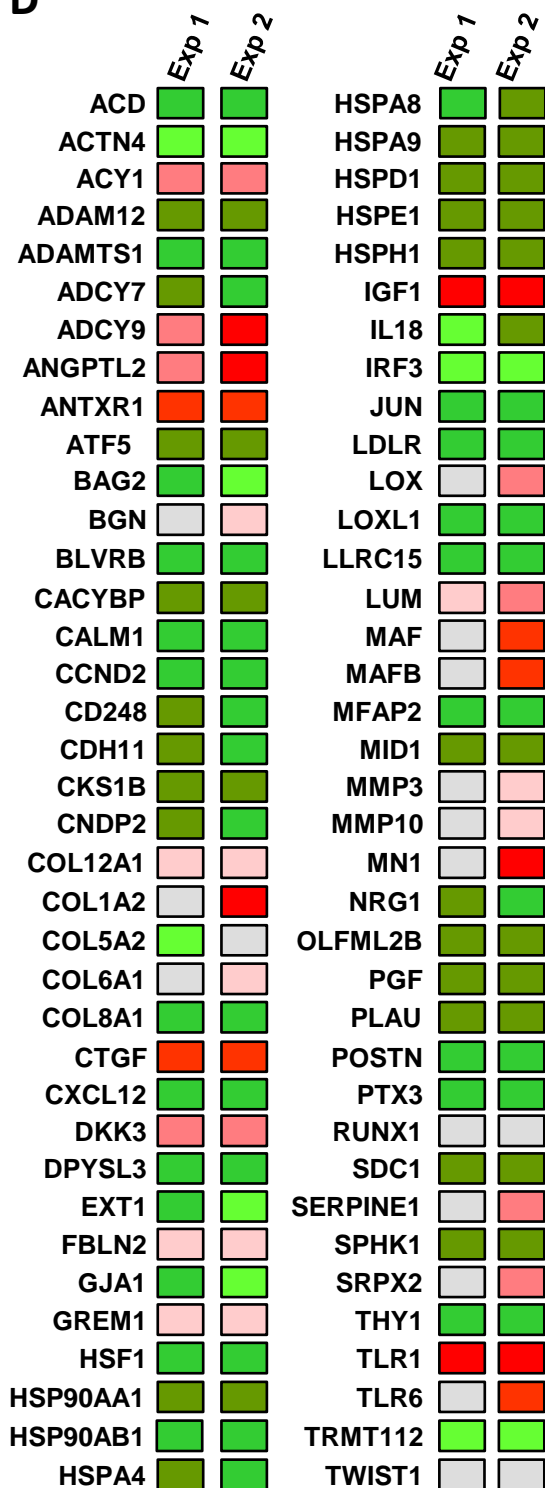
control and patients fibroblasts after 4 days cultured in DMEM 2% FCS, n=3. (C) Western blot showing expression of CCND2, P-AKT and total AKT in patient's fibroblasts compared to controls. Vinculin was used as loading control. (D) Quantification of the ratio phosphorylation/total of p70S6K after Bioplex analysis on cultured fibroblasts, n=2. Cells were cultured in DMEM 2% FCS during one week before quantification. (E) Western blot showing increased phosphorylation of HSF1 and AKT in patient's fibroblasts compared to controls. Vinculin was used as loading control. (F) HSF1 immunofluorescent staining showing nuclear localization of P-HSF1 in patient A's fibroblasts compared to control. P-HSF1 was stained in green and nuclei in blue with DAPI. (G) QPCR analysis of *HSPA4*, *HSPD1* and *HSPE* mRNA (known to be regulated by HSF1) in patient A's fibroblasts and control donors 1 and 2 fibroblasts (one representative experiment from 2). P was determined in comparison to the mean of mRNA from control donors 1 and 2 for each gene. ** P<0.01.

Figure 2: AKT phosphorylation induces HSF1 activity in *PIK3CA* mutated patients. (A) Insulin treatment induces AKT and HSF1 phosphorylation. Cells were starved during 5h then treated for 15 min with 10 nM insulin, lysed and analyzed with Western blotting. Actin was used as loading control, n=2. (B) Inhibition of AKT phosphorylation by LY294002 impairs HSF1 phosphorylation. Cells were treated with 10 μ M LY294002 or 100 ng/ml rapamycin for 7 hours or overnight. Vinculin was used as loading control, n=3. (C) Rapamycin and LY294002 treatment inhibit both cytoplasmic and nuclear HSF1 localization. Cells were treated overnight with 10 μ M LY294002 and 100 ng/ml rapamycin, before P-HSF1 analysis by Western blotting. HSP60 was used as specific cytoplasmic marker confirming the good cytoplasmic/nuclear fragmentation and loading control. Actin was used as loading control, n=3. (D) QPCR array analysis of 74 genes belonging to the HSF1 cancer related-signature in patient A's fibroblasts after 10 μ M LY294002 overnight treatment. Color code was used to

illustrate the gene mRNA down- or up-regulation after LY294002 treatment compared to untreated cells, n=2.

Figure 3: Specific HSF1 inhibitors limit the proliferation of *PIK3CA* mutated cells. Cell growth and viability measured by XTT staining after 48 hours of treatment with (A), dilution medium, 10 μ M LY294002, increasing doses of Triptolide, and (B) dilution medium, 4 ng/ml rapamycin, or increasing doses of Kribb11, n=3. For both experiments : \square control 1, \square control 2, \blacksquare patient B, \blacksquare patient A. Statistical differences are determined for each donor between medium and treatment. (C) Absolute cell count of patient A fibroblasts after 48 hours of treatment or not (medium) with 100 ng/ml rapamycin, 3 μ M Kribb11, 10 nM Triptolide, statistical differences are determined between treatment and medium, n=2. (D) patient A cell growth and viability measured by XTT staining after 48 hours of treatment with Kribb11 or BYL719, n=3. (E) Cell proliferation of fibroblasts from patient A measured by BrdU incorporation using a colorimetric cell proliferation ELISA kit. (F) Inhibition of CCND2 expression in cells from patient A in the presence or not (untreated) of 3 μ M Kribb11, or 10 nM Triptolide, n=3. (G) Cell apoptosis measured by a cell death detection ELISA kit after 24 hours of treatment with 100 ng/ml rapamycin, 10 μ M LY294002, 3 μ M Kribb11, 10 nM Triptolide. 1 μ M staurosporine was used as an apoptosis positive control, n=3. *P<0.05, **P<0.01, ***P<0.005



A**B****C****D****Figure 2**

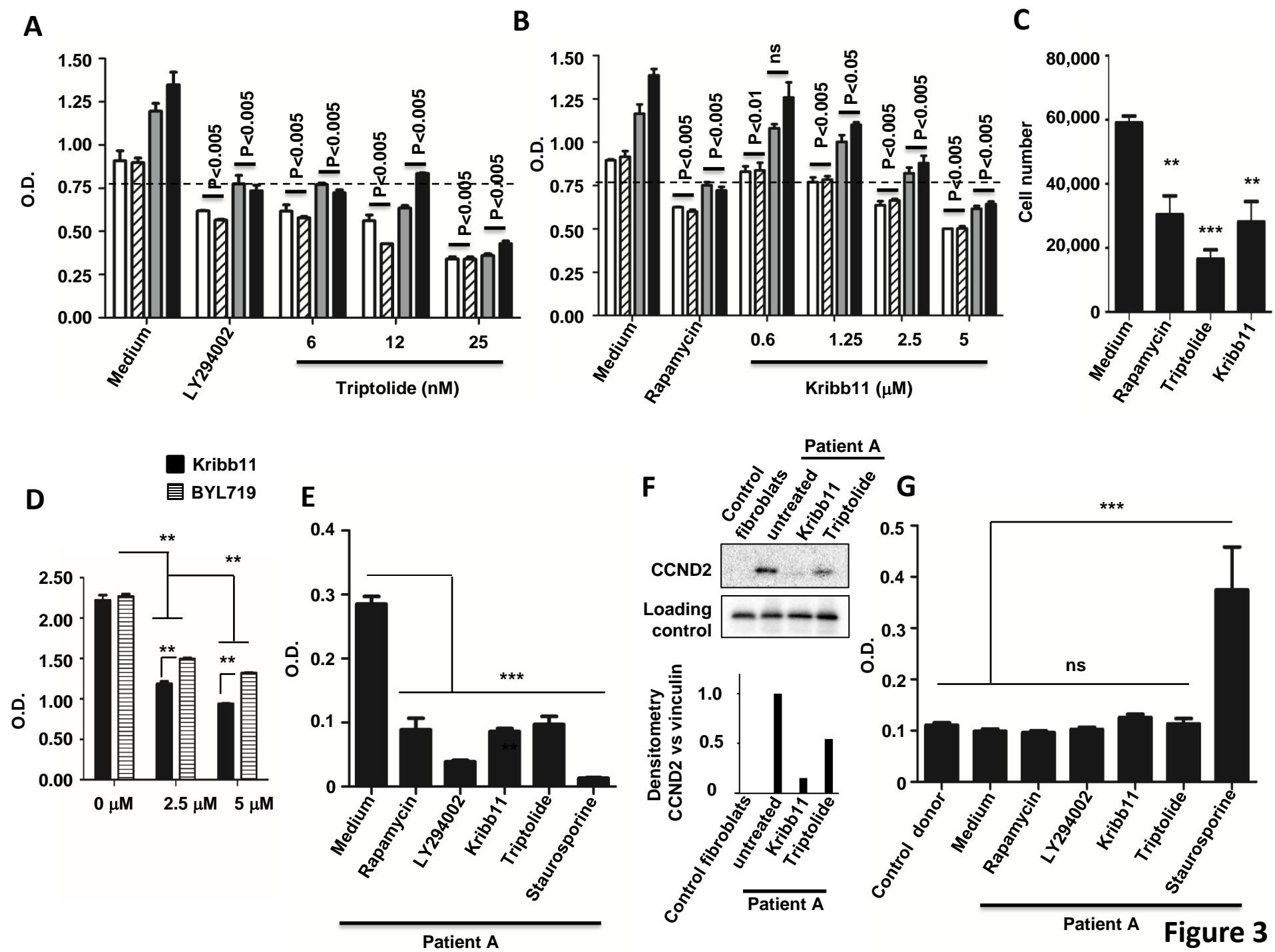


Figure 3