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Multi-tasking Sulf1/Sulf2 enzymes do not only facilitate extracellular cell signalling but also participate in cell cycle related nuclear events

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ABSTRACT:

This study demonstrates highly dynamic spatial and temporal pattern of SULF1/SULF2 expression in a number of neuronal cell types growing in normal culture medium that included their transient nuclear mobilisation. Their nuclear translocation became particularly apparent during cell proliferation as both SULF1/SULF2 demonstrated not only cell membrane associated expression, their known site of function but also transient nuclear mobilisation during nuclear cell division. Nuclear localisation was apparent not only by immunocytochemical staining but also confirmed by immunoblotting staining of isolated nuclear fractions of C6, U87 and N2A cells. Immunocytochemical analysis demonstrated rapid nuclear exit of both SULF1/SULF2 following cell division that was slightly delayed but not blocked in a fraction of the polyploid cells observed in C6 cells. The overexpression of both Sulf1 and Sulf2 genes in C6 and U87 cells markedly promoted *in vitro* growth of these cells accompanied by nuclear mobilisation while inhibition of both these genes inhibited cell proliferation with little or no nuclear SULF1/SULF2 mobilisation. SULF1/SULF2 activity in these cells thus demonstrated a clear co-ordination of extracellular cell signalling with nuclear events related to cell proliferation.

Highlights:

- Dynamic SULF1/2 mobilisation to nucleus during cell cycle
- SULF1/2 co-ordinate extracellular cell signalling with nuclear role in cell cycle
- SULF1/2 overexpression increases cell proliferation while their inhibition reduces growth

Keywords: Sulf1, Sulf2, cell cycle, nocodazole, nuclear mobilisation

Introduction:

Sulfatase enzymes constitute a large family of proteins detected in many tissues many of which are located in the lysosome within the cell [1-3]. Lysosomal sulfatases cleave a range of sulfated carbohydrates including sulfated glycosaminoglycans and glycolipids. Sulfatases play important roles including the degradation of sulfated glycosaminoglycans and glycolipids in the lysosome, and in remodelling of sulfated glycosaminoglycans in the extracellular space. In addition to the intracellular sulfatases, later studies also detected a new family of sulfatases called Sulf1 and Sulf2 that from their molecular structure could be predicted to have an extracellular

function [4, 5]. While the catalytic region including the catalytic site of the new sulfatases showed high homology to most previously described intracellular sulfatases, SULF1/SULF2 enzymes in contrast included not only a hydrophobic signal for secretion but also a large segment of highly basic hydrophilic domain ideally suited to dock such enzymes to the cell membrane [4, 5]. Their extracellular function became further apparent from the extracellular localisation of these enzymes on the cell membrane. Their function on the cell membrane was soon discovered to relate to cell signalling events as these enzymes promoted some cell signalling pathways requiring desulfated HSPG co-receptor function to facilitate ligand receptor interaction while inhibiting those cell signalling pathways requiring sulfated HSPG function [6-8]. Catalytic analyses showed SULF1/SULF2 to specifically desulfate 6-O sulfates required for ligand receptor interaction of certain growth factors e.g. FGF, VEGF signalling that could thus be inhibited by these enzymes. The activities of other growth factors/signalling molecules sequestered in ECM in contrast can be promoted by their release from ECM by SULF1/SULF2 desulfation to faciltate ligand receptor interaction. Membrane associated SULF1/SULF2 expression and their cell signalling function has been confirmed by many research groups. Our recent study, however, also detected their expression in the nuclei of some normal *in vivo* neuronal cells [9] and some cancer cells (unpublished). Our *in vitro* analysis of some neuronal cell lines further confirmed SULF1/SULF2 expression in a proportion of the nuclei of such cells. SULF1/SULF2 expression in the nucleus of such cells appeared highly dynamic and transient which in this study was found to relate to nuclear cell division. The levels of SULF1/SULF2 expression were markedly reduced in high density cell cultures with non-detection in the nucleus. The precise levels of SULF1/SULF2 expression in a cell thus were highly variable during different phases of growth and corresponded to the specific phase of cell proliferation.

Materials and Methods:

Cell culture, transfection and nocodazole block: All cells (C6, U87, N2A and SH-SY5Y) obtained from ATCC were grown in Dulbecco's modified Eagle's (DMEM) medium with 10%FCS. Human Sulf1 and Sulf2 cDNA constructs cloned in pcDNA3 described previously [10-12] were transfected into C6 and U87 cells for overexpression analysis. Sulf1 and Sulf2 were transfected with an EGFP expression vector while the control cells were transfected with EGFP/pcDNA3 alone using the Bio-Rad TransFectin reagent. To enrich the mixed population of transfected cells, the normal growth medium was changed to DMEM/10%FCS with 800µg/ml G418 following 48 hour of growth in the normal medium. RNAi vectors for Sulf1/Sulf2 inhibition in U87 cells were constructed using the shRNAi.2 (Clonetech) vector by the addition of hairpins targeting 240bp-exon 6 of Sulf1: AAACGAGAAAGATTATGGAAC; and 240bp-exon 6 of Sulf2: TACGTCCACAACCACAACACC. These were then co-transfected along with pcDNA3.1 to confer G418 resistance. Transfected cells were allowed to recover and selected in the presence of G418 for 3 weeks. Neutralisation antibodies at 200ng/ml antibody to SULF1 (C) or SULF2 (D) were used to inhibit SULF1/SULF2 in C6 cells as previously described [12]. Transfected and si-RNA inhibited cells grown on Lab-Tek II chamber slides following 3 weeks of *in vitro* growth in G418 containing medium as well as antibody treated cells were used for SULF1/SULF2 immunocytochemical staining.

To synchronise cell division *in vitro*, C6 cells following 1 day growth *in vitro* were treated with 200ng/ml nocodazole for 16 hours to arrest cells at G2/M stage before washing the cells with the fresh culture medium to remove all trace of nocodazole before replacement with 10%FCS DMEM to follow recovery. The cells were fixed after different time intervals to follow recovery.

Cell Synchronisation and Flow Cytometry: C6 cells were cultured in complete medium for 24 hours before being treated with 200 ng/ml nocodazole for 16 hours to block cells in the G2/M stage of the cell cycle. The following day, cells were released from the block and allowed to continue through the cycle via addition of fresh media. Cells were then collected at various time points of 0, 1, 3, 4, 6, 8, and 24 hours following release from block in conjunction with an asynchronised control which consisted of untreated cells. At each time point, cells were harvested by trypsinisation followed by addition of 2 ml of fresh media and cell suspensions transferred to FACS tubes. Cell suspensions were transferred to 3ml round-bottom FACS tubes (Invitrogen) and centrifuged (500g, 5 min, 4°C). Cell pellets were washed in 1 ml PBS and were resuspended in 300 µl of PBS and gently vortexed while adding 700µl of ice-cold 70 % ethanol drop-wise to the cell suspension. After fixation, the cell pellets washed once with PBS, and resuspended by vortexing in a propidium-iodide staining buffer (10mM Tris PH 8.0, 150mM NaCl, 0.1% NP- 40, 10 µg/ml RNAseA, 50µg/ml propidium Iodide) for 45 min at 4^{o}C in the dark. Following incubation, cells were centrifuged at 500g for 5 min and resuspended in 300µl PBS before DNA content was determined using a BD Canto II analyser with PI detected in the phycoerythrin (PE) channel with linear amplification and a total of 10,000 events collected for each sample. Data was plotted on a dot plot where each individual dot represented a single event or cell and a single population of cells was selected by drawing a box or gate around the cells. This population was used to generate a histogram to compare to DNA content (x-axis) with number of cells or events (y-axis). The resulting graph is a cell cycle profile where the different phases of the cell cycle, namely G0/1, S and G2/M were identified by the peaks and troughs. The percentage of cells in each phase of the cell cycle was analysed using ModFit LT software, USA.

Cell Fractionation: Cell fractionation was performed essentially as described before [13]. Briefly, six 10 cm plates of cells were grown to about 80% confluence. Cells were collected by scraping, gently centrifuged, washed with hypotonic buffer [20mM Tris (pH 7.5), 3mM $CaCl_2$, and 2mM $MgCl_2$], and then resuspended in 5 packed cell volumes of hypotonic buffer and incubated on ice for 20 min. Swollen cells were homogenized in a Dounce homogenizer and centrifuged at low speed (500g) to pellet crude nuclei. The postnuclear supernatant was further processed as described below. The crude nuclear pellet was further purified by resuspension in 500µl ER stripping buffer [10 mm Tris (pH 8.0), 3mM $CaCl_2$, 2mM $MgCl_2$, 0.1mM EDTA, 1mM dithiothreitol, and 0.5% Nonidet P-40], incubated on ice for 15 min, and then centrifuged at 500g for 5 min. The supernatant containing ER fragments originally associated with nuclei is combined with membrane pellet below. Purified nuclei were loosened by tapping and mixed with 1/3 packed cell volume high salt buffer [420mM NaCl and 20mM Tris (pH 7.5)] with for 30 min at 4^{o}C. The resulting suspension was centrifuged for 20 min at 4^{o}C at 16000g, and the supernatant, containing soluble nuclear proteins, was collected, diluted with an equal volume of hypotonic buffer, and

collected as the nuclear fraction. The postnuclear supernatant obtained above was centrifuged at 16,000g for 20 min at 4°C. The supernatant was collected as the cytosolic fraction, and the pellet was collected as the crude ER/membrane fraction and re-suspended in the ER supernatant as collected above. All buffers contained a protease inhibitor cocktail (Complete Mini, Roche). Cytosolic, ER/membrane, and purified nuclear fractions were analyzed by western blotting using SULF1 and SULF2 antibodies.

Immunocytochemical staining of cells: The pattern of SULF1/SULF2 expression in cell cultures was examined using rabbit peptide antibodies C (1/200) and D (1/100) to these enzymes described previously [10, 11, 14, 15]. The binding of primary antibodies was detected using goat anti-rabbit biotinylated IgG (1/400) followed by streptavidin conjugated Alexa Fluor 488 fluorochrome (1/400). The expression of tubulin using double immunofluorescence procedure was detected by Abcam rat anti tubulin (1/200) visualised by anti rat immunoglobulins linked to Alexa Fluor 594 fluorochrome (1/400). Cells treated with pre-immune rabbit sera were similarly incubated with fluorochrome-labelled secondary antibodies as controls (not shown). Cells with primary antibody dilutions were incubated overnight at 4°C followed by PBS rinses and secondary antibody incubations for 1hour each at room temperature. Antibody-labelled cells were mounted in fluorescent mounting medium (Sigma Aldrich) containing 2.5µg/ml DAPI for nuclear visualisation and photographed using a Leica DM4000B fluorescent microscope. Quantification of the individual fluorescent images was carried out using Volocity software by measuring average pixel count/image.

RESULTS:

1. Highly dynamic spatial and temporal expression of SULF1 and SULF2 in neuronal cells: A proportion of four neuronal cell lines C6,N2A, SHSY5Y and U87 tested in this study did not only show the expression of SULF1 and SULF2 proteins in cytoplasm, its site of translation or the cell membrane, its site of cell signalling facilitation but also in a number of the nuclei (Figure 1) the proportion of which varied in different cell cultures. Further immunocytochemical analysis of C6 cell cultures indicated spatial and temporal changes in SULF1 and SULF2 expression to relate to the cell density at any given stage (Figure 2). The relationship to cell density became very apparent when the cells seeded at different densities showed preferential nuclear or membrane or both nuclear and membrane associated SULF1/SULF2 expression. For example, nuclear SULF1/SULF2 expression was usually observed during earlier stages of growth when the cells were actively proliferating. In C6 cells, maximum nuclear expression of SULF1/SULF2 was observed in nascent daughter nuclei pair with the elongated thinner shape and size of the nuclei being much smaller at this stage, as seen by DAPI staining (Figure 2). The area of SULF1/SULF2 expression during cell division usually extended into the perinuclear region but not into peripheral cytoplasm. The nuclear SULF1/SULF2 expression, however was transient as the SULF expression in the nucleus was rapidly down-regulated following subsequent cellular growth. The SULF1/SULF2 expression downregulation in the nucleus always first became apparent in the centre of the nucleus followed by down-regulation in the peripheral nucleus. SULF1/SULF2 proteins became undetectable in the nuclei soon after cell division and as the cell cultures became confluent. The expression of these enzymes in high density

cultures was thus usually restricted to only the cell membrane with little or no expression in the cytoplasm or the nuclei (Figure 2). The correlation of SULF1/SULF2 translocation into cell nucleus with cell cycle was further highlighted by their correlation with expression of tubulin (Figure 3). Tubulin is a component of microtubules involved in the assembly of a mitotic spindle that ensures proper attachment of microtubules to chromosomes through kinetochores before cells enter anaphase and can segregate into daughter cells (Figure 3). The cells for this experiment were treated with nocodazole for 16 hours and analysed at different time points following recovery. Nocodazole reversibly arrests cell cycle as it disrupts microtubule function by binding to β-tubulin. It suppresses microtubule dynamics by inducing microtubule depolymerization. The levels of SULF1 and SULF2 closely correlated with the levels of tubulin during nuclear localisation (Figure 3).

2. Cell fractionation confirms SULF1 and SULF2 expression in the neuronal cell nuclei: Since the SULF1/SULF2 enzymes are known to facilitate cell signalling on the cell membrane and their expression in the nucleus was unexpected, we analysed the association of these enzymes with the nucleus further by immunoblotting analysis of membrane, cytosol and nuclear fractions of not only the C6 but also U87 and N2A cells. The immunoblotting analysis of C6 cell fractions showed no SULF1 or SULF2 expression in the soluble cytosol but the staining of two SULF1 as well as SULF2 bands in the cell membrane. The molecular weights of two SULF1 bands were about 60 and 40kD while the size of membrane associated SULF2 was 70 and 45kD. Unlike the membranous fraction the two major bands stained with the SULF1 antibody in the nucleus were of 125kD and 50kD although some weak staining was also observed with some other minor bands of intermediate mobility. As was the case with C6 cells, no SULF1 or SULF2 expression was observed in the U87 cell cytosol but the SULF2 staining of the U87 membrane fraction showed the presence of two major bands of 70 and 50kD with a weaker staining of the 75kD. SULF2 in the nucleus also showed the presence of these two major bands and low levels of 100kD, 45kD and 33kD. SULF1 staining of U87 membrane fraction similarly showed the presence of two major bands of 70 and 48kD but also a weak staining of an additional smaller size band of 30kD. The U87 nuclear fraction in contrast did not only show the presence of two major bands associated with the cell membrane but also low level expression of additional bands (125kD, 100kD and 30kD). Immunoblotting of N2A cell fractions showed no SULF1 expression in the cytosol but dark staining of 4 major bands of 125, 75, 40 and 20kd sizes in the membrane fraction. While the level of SULF1 expression in the nuclear fraction was lower than in the membrane fraction, dark staining of three major bands of 72, 28 and 18kd was observed in the nuclear fraction that also included significant staining of a 125kD band. Immunoblotting of N2A cell membrane fraction with SULF2 antibody showed dark staining of 3 major bands of 72, 43 and 33kd molecular sizes that unlike the C6 and U87 cells also showed some expression of three SULF2 bands of 74, 63 and 33kD sizes in the cytosol fraction. N2A Nuclear fraction also showed a weaker but significant staining of a number of bands in similar molecular range although the darkest staining band was of 28 or 30kD. Nuclear SULF1/SULF2 expression was thus not only apparent by immunocytochemistry but also by immunoblotting procedure. The SULF1/SULF2 profiles in the nuclei were more complex than their expression on the cell membrane indicating diverse or different role in the nucleus. The difference in molecular sizes may relate to further post-translational processing of these proteins [16].

3. SULF1 and SULF2 expression is closely associated with the cell cycle: Since the distribution and level of SULF1/SULF2 expression appeared to vary from culture to culture, we next analysed the expression of these enzymes in synchronised cell cultures following a 16 hour mitotic block with nocodazole to investigate the changes in SULF1/SULF2 expression following release from the mitotic block. The nocodazole block at G2M phase of cell cycle led to a marked reduction in both SULF1/SULF2 expression but the levels of these enzymes gradually increased following recovery (Figures 5-7). A number of cells showed nuclear SULF1/SULF2 expression during early stages of recovery but with little or no nuclear SULF1/SULF2 expression after 8 hours when the levels of these enzymes even on the cell membrane had decreased (Figures 6 & 7). While flow cytometric analysis (Figure 5) showed the largest proportion of cells going through mitosis during earlier phase of 1-2 hours, the maximum SULF1/SULF2 expression in these cell cultures was observed at 4 hours recovery using immunocytochemical quantitation by measuring pixels/area that does not distinguish between nuclear and cytoplasmic or membranous expression. A number of cells, however, were observed with nuclear SULF1/SULF2 staining even at 4-hour recovery time. The highest level of cytoplasmic SULF1/SULF2 expression usually related to the larger size of the cells (Figures 6&7). Compared to 4 hour stage, only a small proportion of the cells showed larger size associated with darker cytoplasmic staining of SULF1 as well as SULF2 at 8 hours while the SULF1/SULF2 staining was generally restricted to only the cell membrane by 24 hours. It is also clear that a proportion of the cells expressed no SULF1 or SULF2 during any stage of growth. This analysis of nuclear expression thus showed transient and highly dynamic relationship to cell cycle and the maximum level of nuclear SULF1/SULF2 expression appeared to relate to G2M stage of cell cycle. Also apparent from this analysis was the increased expression of both SULF1/SULF2 during increasing cell size of C6 cells. The variation in cell size during cell cycle was also apparent from the H&E staining of the C6 cells (Figure 8). The presence of dividing cells during recovery following nocodazome treatment was also apparent using the routine H&E stain. Quantitative changes in cells undergoing cell cycle following G2M block during different stages of recovery became apparent from the H&E analysis showing 0.63%$\pm$0.29% cells dividing at 0 hour, 3.38%$\pm$0.22% at 2 hours, 2.91%$\pm$0.30% at 4 hours, 1.28%$\pm$0.25% at 8 hours and 0.31%$\pm$0.16% cells dividing at 24 hours. The H&E stained cells were also used to determine the changes in cell size during recovery time following 16-hour nocodazole treatment (Figure 8) that showed cell size increase at 2, 4 and 8hours when compared with 0hr and 24hr.

4. Nuclear expression of SULF1/SULF2 is prolonged in polyploid neuronal cells Since the SULF1/SULF2 expression in the nucleus appeared downregulated following mitosis, we speculated that the expression of these enzymes in the nuclei of polyploid cells that fail to proceed to cytokinesis may continue to show their nuclear expression of these enzymes. The flow cytometric analysis indicated the presence of a fraction of C6 polyploid cells that light microscopy revealed to be large irregularly shaped cells with irregularly shaped nuclei becoming most apparent at 4 hours of culture. We therefore examined the expression of SULF1/SULF2 in such cells at 4 hour recovery time following nocodazole treatment. The level of nuclear SULF1/SULF2 expression in such polyploid cells appeared to be generally higher as indicated by darker staining of these cells (cells indicated by arrows in Figure 9). While a large majority of diploid cells with single nucleus at 4 hours showed little or

no SULF1/SULF2 expression, these enzymes persisted in the nuclei of polyploid cells for longer than in the normal diploid nuclei as their expression was still apparent at 4-hours unlike most diploid cells. SULF1/SULF2 expression even in polyploid cells, however, was down-regulated gradually and thus did not relate to cytokinesis event since SULFs were eventually eliminated from the nuclei despite the failure of nuclear segregation.

5. SULF1/SULF2 overexpression promotes while SULF1/SULF2 silencing inhibits cell division and their nuclear mobilisation: Since both SULF1 and SULF2 showed marked changes during cell division and cell growth, we further investigated the role of increased ectopic SULF1/SULF2 expression as well as their inhibition by siRNA knockdown in U87 and using neutralisation antibodies in C6 cells. The ectopic expression of both SULF1 and SULF2 in U87 as well as C6 cells showed a significantly higher rate of cell proliferation and detection of increased number of cells showing nuclear SULF1/SULF2 expression (Figure 10). The overexpression of SULF1/SULF2 genes thus did not only show increased number of cells with nuclear expression of these enzymes but also, importantly, increased cell proliferation measured by increased cell number. This was markedly different when SULF1/SULF2 expression was inhibited by siRNA in U87 and C6 cells. Most such cells following siRNA knockdown showed little or no SULF1/SULF2 expression in the nuclei and only a low level expression restricted to only the cell membrane. Only a rare cell in such siRNA treated cultures showed any nuclear expression of SULF1/SULF2 (Figure 10). Moreover, cellular proliferation was inhibited in these cells.

DISCUSSION:

It is now well established that both SULF1/SULF2 enzymes are located on the cell membrane where they facilitate cell signalling by promoting ligand receptor interaction through regulated de-sulfation of HSPGs [4, 5]. Their extracellular location was also apparent from their modified gene structure that did not only include a hydrophobic signal for secretion but also the presence of a highly basic hydrophilic domain at the C-terminal end that is lacking in all the intracellular sulfatases [4, 5]. This highly basic hydrophilic domain is ideally suited to dock these SULF enzymes to the cell membrane. Their site of action therefore was assumed to be entirely extracellular on the cell membrane. Our earlier study of some neuronal tissues, however indicated the expression of these enzymes and particularly of SULF2 in the nuclei of some neuronal cells [9]. The present study confirms such unexpected localisation of both SULF1 and SULF2 enzymes in the nuclei of all four neuronal cell lines tested in this study. While SULF1/SULF2 enzymes on the cell membrane are known to desulfate HSPGs to facilitate certain cell signalling pathways the role of these enzymes in the nucleus is not known. HSPGs, however, are known to be expressed in the nucleus in both sulphated and desulfated forms where they have been proposed to have multiple regulatory functions in the cell cycle, transcription and transport of cargo to the nucleus [17]. The differential levels of nuclear HSPG sulfation during all these nuclear activities [17] including cell cycle were thus attributed to the activities of different sulfotransferases. The present study demonstrates further editing of sulfotransferase regulated HSPG sulfation not only

extracellularly on the cell membrane or ECM but also intracellularly in the nucleus during cell cycle.

The present study shows that the level of SULF1/SULF2 expression *in vitro* growth of C6 cells was not static and not only quantitatively variable but also spatially dynamic as their localisation varied markedly during different stages of growth. Comparing quantitative levels of these enzymes without specifying the cell density or different stages of growth can thus be misleading as their levels continuously change during growth and varied culture conditions impacting growth. The unexpected observation in this study was the SULF1/SULF2 expression in the nuclei. The changes in SULF1/SULF2 expression may relate to changes in cell signalling promoting growth or some transcriptional activity. It is clear that cell signalling induced cell proliferation at least in some cells also extends to cell cycle itself. What precise role the SULFs play in the nucleus is not clear but could desulfate HSPGs in the nucleus not only to facililtate cell proliferation but also to promote transcription or gene expression as level of HSPG sulfation has been demonstrated to impact all these activities. SULF1/SULF2 enzymes could thus regulate cell cycle as desulfation by these enzymes could regulate gene expression through regulation of transcription since HSPG sulfation is known to inhibit histone acetylation. Acetylation of histones increases gene expression by transcriptional activation while deacetylation leads to reduced gene transcription. Nuclear HSPG desulfation by SULF1/SULF2 enzymes could thus increase transcription.

The composition of HSPG and its sulfation status in the nucleus has the potential to influence cell division in a positive or a negative manner. For example, a higher level of sulfation in the nucleus has been reported once the cells reach confluency in certain cell types [18] although this may not apply to all cell types. The downregulation of SULF1/SULF2 in the nuclei of C6 confluent cells and also their much reduced expression in the cytoplasm and the cell membrane in such high density cell cultures would also permit higher levels of sulfation that agrees with increased sulfation in the absence of de-sulfating enzymes during the lag/stationary phase of this cell line. Down-regulation of SULF1/SULF2 enzymes leading to increased levels of sulfation thus can decrease or prevent cell proliferation and may explain why high levels of these enzymes are usually detected during fetal growth of most tissues with a few exceptions such as some neuronal and skeletogenic tissues [9, 19].

High levels of both SULF1/SULF2 in the nucleus were observed during cell division in the present study that would induce decreased nuclear levels of HSPG sulfation thus enabling the cell cycle to progress through S, G2 and M phases. This further confirms that some cell types undergoing cell division reduce nuclear HSPG sulfation and could upregulate sulfation following next round of cell cycle. The SULF1/SULF2 expression in the nucleus was thus highly dynamic showing a clear correlation between cell cycle and nuclear localization of these enzymes. The level of SULF1/SULF2 expression, however, was rapidly downregulated in the nucleus following cell division but SULF1/SULF2 suppression in the nucleus was not strictly related to cytokinesis since even the polyploid cells show slightly delayed but eventual downregulation of these enzymes similar to the diploid cells.

Following cell division, the newly formed cell can prepare for next round of cell cycle by growing larger in size to accumulate cell mass, chromosomal DNA duplication and preparation for mitotic spindle during G1 but also some further growth taking place in size during S and G2 phase before undergoing a next cycle of mitosis ensuring that the cell size is not diminished with each cell cycle. This was clearly apparent from the cell size change observed following peak of cell division that was particularly apparent at 4 hours of recovery following nocodazole block of C6 cell mitosis. It is not, however, clear why a high level of both SULF1 and SULF2 accumulation was observed in the cytoplasm at this stage and whether it was related to some downstream intracellular signalling of extracellular mitogen promoted proliferation or its participation in mTOR cell signalling. It is not clear if this results from some size sensor to determine if the cells are sufficiently large to commit to cell division and whether SULFs are involved in promoting cell size increase as indicated by their high level of cytoplasmic expression following cell division. It is possible that cells have an intracellular mechanism to sense environmental growth conditions. If conditions permit growth, the signalling centre will trigger mass accumulation by initiating synthesis of new protein. TOR is a central component of this signalling centre and is directly responsible for activating the protein synthesis machinery. The reason why high levels of SULF1/SULF2 expression at 4 hours may relate to cell signalling pathways involved in increasing cell size *via* mTOR pathway is not clear.

The changes in SULF1/SULF2 in response to cell cycle show some similarity to changes in Hippo pathway. For example, cells that are preparing for a next round of cell cycle are inhibited by the hippo pathway that is inhibited by increased cell number and therefore abrogating further cell division. Hippo pathway promotes cell cycle but is switched off following cell contact inhibition i.e. when the cell density increases. It is, however not clear whether these two pathways act independently or if they are related to each other.

The highly dynamic spatial and temporal changes of expression during cellular growth thus highlights the multiple tasks of both SULF1/SULF2 enzymes not restricted to only extracellular cell signalling. Importantly, our findings introduce a novel role for these enzymes in cell division although the precise mechanism of their action in the nucleus remains to be determined.

FIGURE LEGENDS:

Figure 1: Immunocytochemical detection of SULF1 and SULF2 proteins in 4 different neuronal cell lines demonstrates their nuclear expression in a sub-set of cells as indicated by arrows.

Figure 2: Immunocytochemical detection of SULF1 in low density (A) and high density (B, C) C6 cell cultures. A,B and C show Sulf1 staining superimposed with DAPI while A1,B1 and C1 show only DAPI staining to highlight the distinctive nuclear morphology following cell division. The nuclear comparison also highlights the broader expression of SULF1 around the nucleus as pointed out by the arrows.

Figure 3: The correlation of SULF1 (A) and SULF2 (D) expression with localisation of tubulin involved in mitotic spindle formation during cell division in C6 cells examined at 4 hours recovery time following cell cycle arrest with nocodazole.

Spindle formation by microtubules in this figure is indicated by arrows and asterisks (B). DAPI stain highlights the normal and abnormal nuclear morphology when nuclei fail to separate in some polyploidy cells (nuclei indicated by arrows and asterisks in C). The cells showing high level of SULF1 and SULF2 nuclear expression correlating with tubulin are indicated by arrow with a circle.**Figure 4**: Identification of both SULF1 and SULF2 in not only cell membrane (M) but also nuclear (Nuc) fractions of C6, U87 and N2A cells with little expression in cytosol (C) using immunoblotting procedure.

Figure 5: **A:** Freshly passaged C6 cells were blocked, 24 hours after culturing in complete growth medium, at the G2/M stage using 200ng/ml of nocodazole for 16 hours before cells were released from the block by addition of fresh media and cells collected at various time-points and DNA content was analysed by flow cytometry after staining with propidium-iodide. Cell cycle profiles at these time points (0, 1, 3 4, 6 and 8hr) are presented as histograms. Asynchronous, normally cultured cells (Asyn) were included as controls. Data shown are representative of three independent experiments with duplicate observations per time-point. **B:** Percentages of cells in different stages of the cell cycle are represented by bar graphs. Average fluorescence of cells stained for SULF2 after nocodazole block and release as in A at the different stages of the cell cycle is presented as bar graphs. **C**: Changes in levels of SULF1 and SULF2 expression measured using volocity software during different time intervals of recovery following 16-hour Nocodazole treatment. (a=p<0.0001, b=p<0.001, c= p<0.01, d=p<0.05)

Figure 6: Changes in levels and spatial expression pattern of SULF1 during different time intervals of recovery following 16-hour nocodazole treatment of C6 cells including an untreated control (C).

Figure 7: Changes in levels and spatial expression pattern of SULF2 during different time intervals of recovery following 16-hour nocodazole treatment of C6 cells including an untreated control (C).

Figure 8: Changes in cell size and cell division using H&E stain during different time intervals of recovery following 16-hour nocodazole treatment of C6 cells including an untreated control (C). Cell size from H&E stained cells was measured using ImageJ software at indicated time points (n= 25 measurements per field, 3 fields per time point). (a, b, c : p< 0001)

Figure 9: SULF1 and SULF2 expression in polyploid cells and those cells in which nuclei failed to move apart show higher level of expression and delayed nuclear suppression

Figure 10: Sulf1 and Sulf2 gene over-expression achieved by transfection increased cell proliferation and the levels of SULF1 and SULF2 protein expression in transfected cells. Sulf1/Sulf2 inhibition using si-RNA transfection for U87 cells and by using SULF1 (C) and SULF2 (D) neutralisation antibodies for C6 cells reduced cell proliferation as also confirmed by reduced levels of protein detected by antibody stain. The histograms demonstrate the quantitative differences in the levels of SULF1 and SULF2 following ectopic over-expression and inhibition as determined by using volocity software. **p=0.001, *p=0.01.

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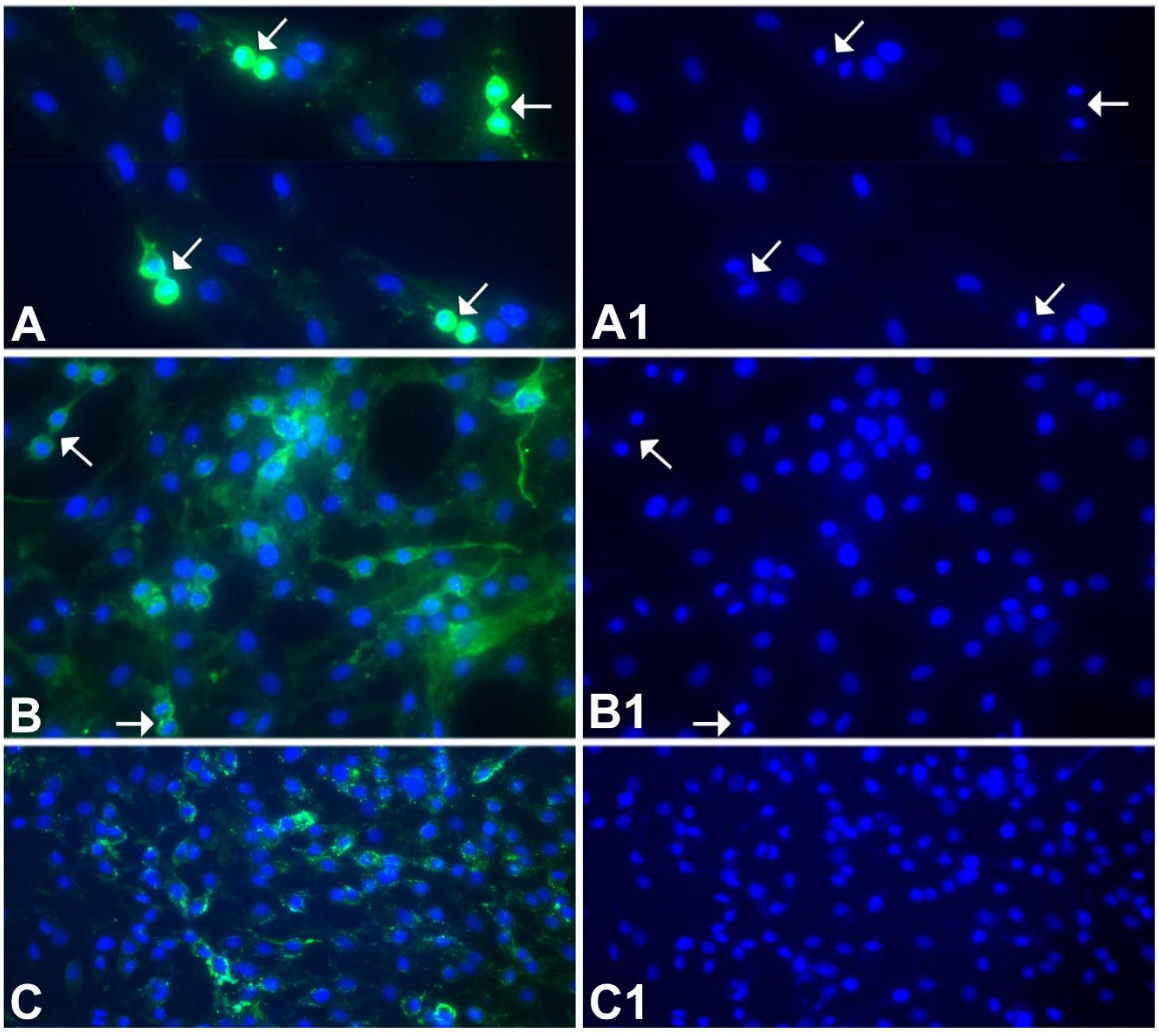
SULF1
SULF2
C6
N2A
SH-SY5Y
U87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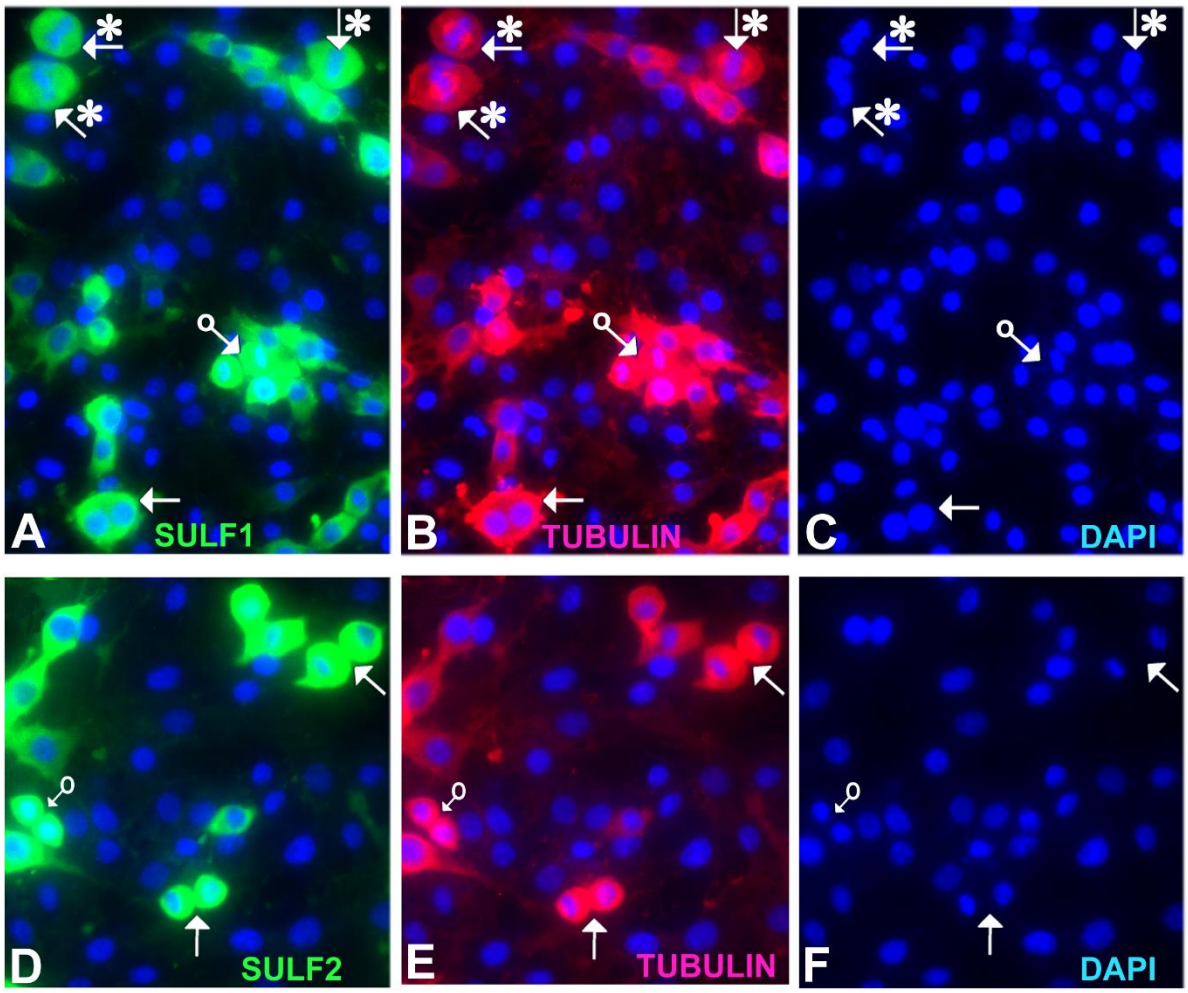
C
C1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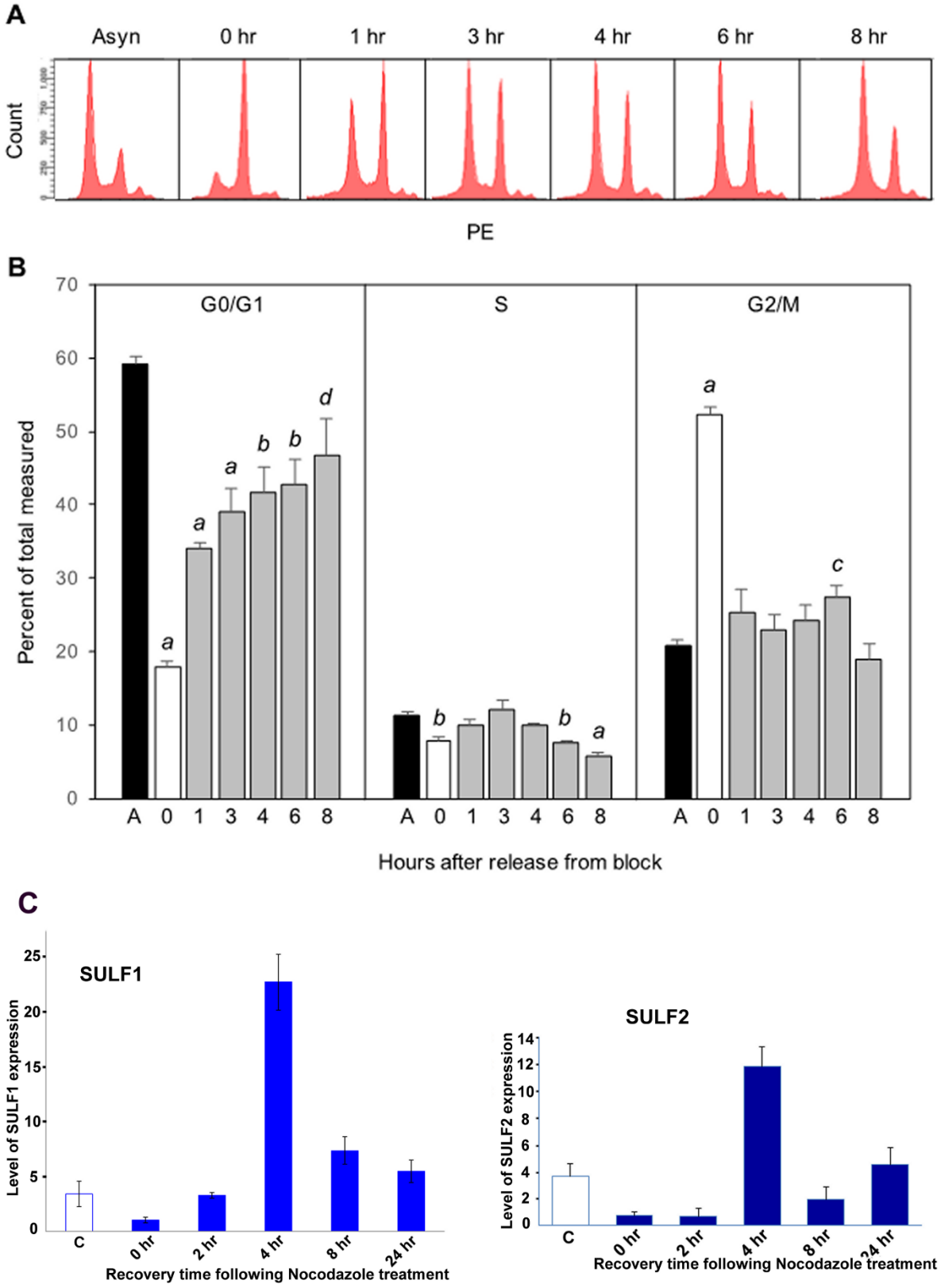
D
SULF2
E
TUBULIN
F
DAPI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Asyn
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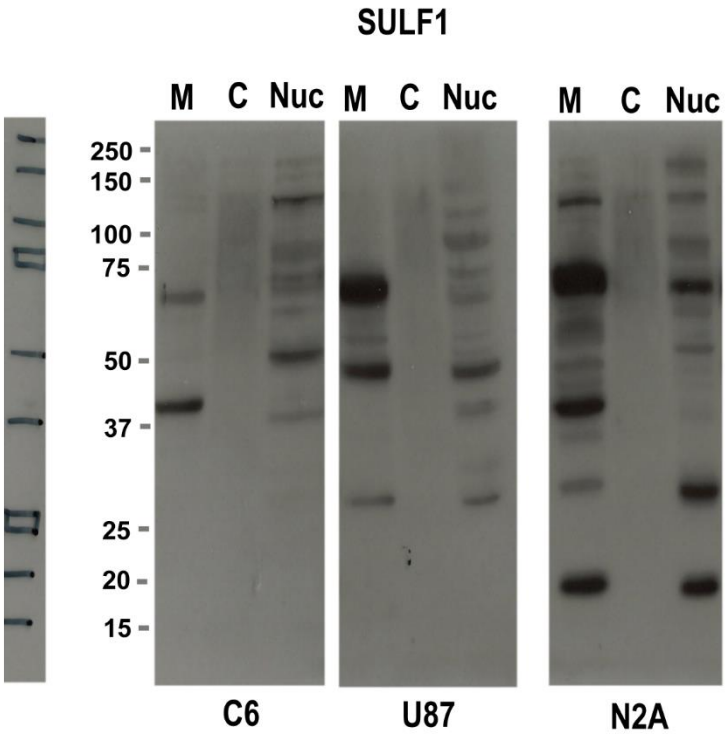
3 hr
4 hr
6 hr
8 hr
Count
PE
B
G0/G1
S
G2/M
Percent of total measured
Hours after release from block
A 0 1 3 4 6 8
C
SULF1
Level of SULF1 expression
C 0 hr 2 hr 4 hr 8 hr 24 hr
Recovery time following Nocodazole treatment
SULF2
Level of SULF2 expression
C 0 hr 2 hr 4 hr 8 hr 24 hr
Recovery time following Nocodazole treatment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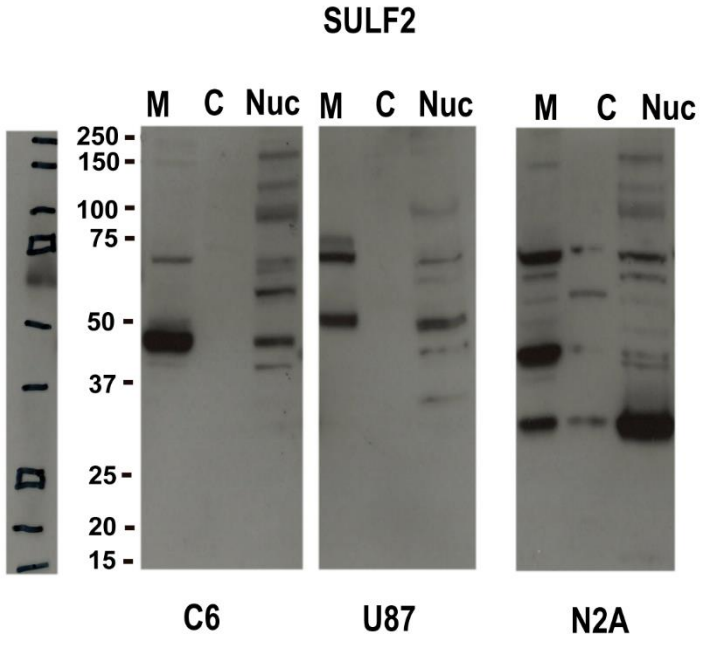
C6
U87
N2A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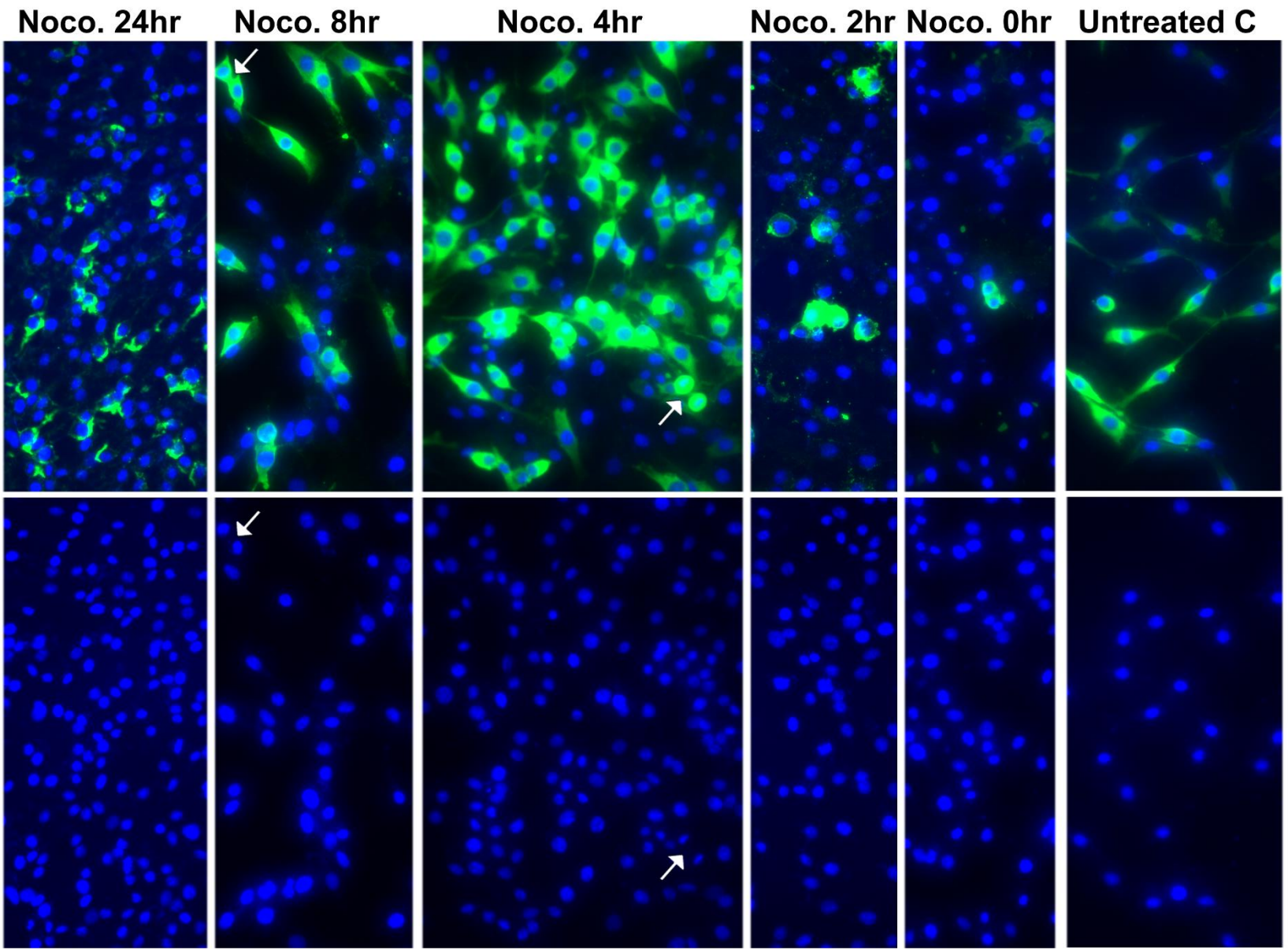
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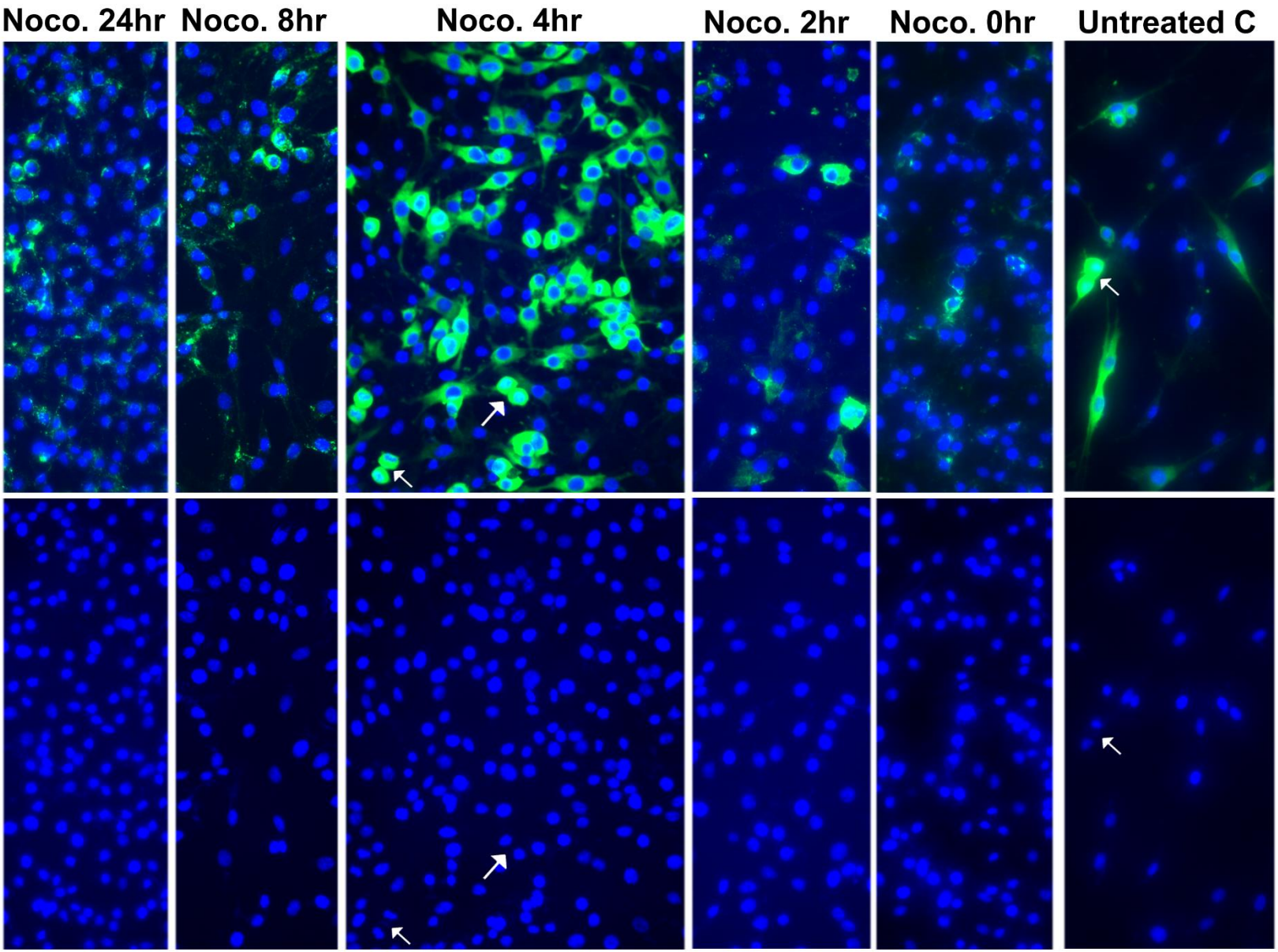
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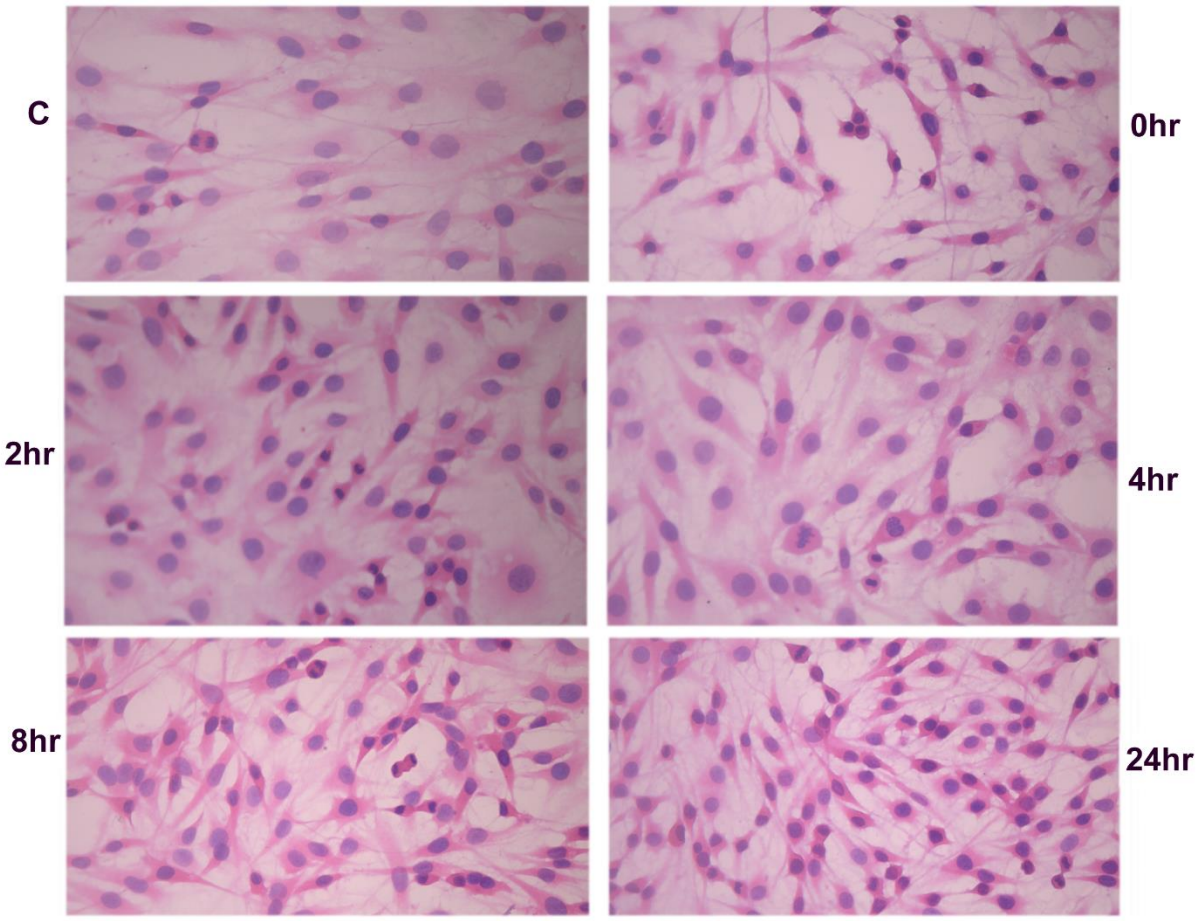
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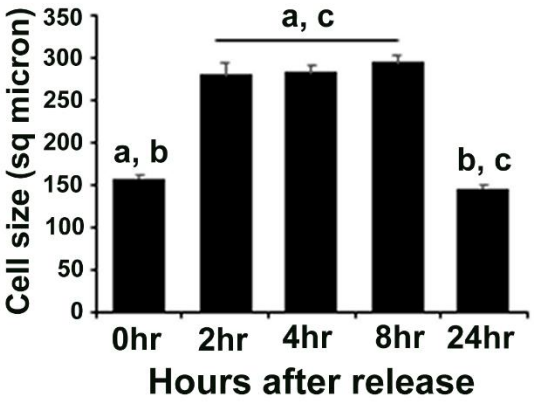
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a, c
a, b
b, c
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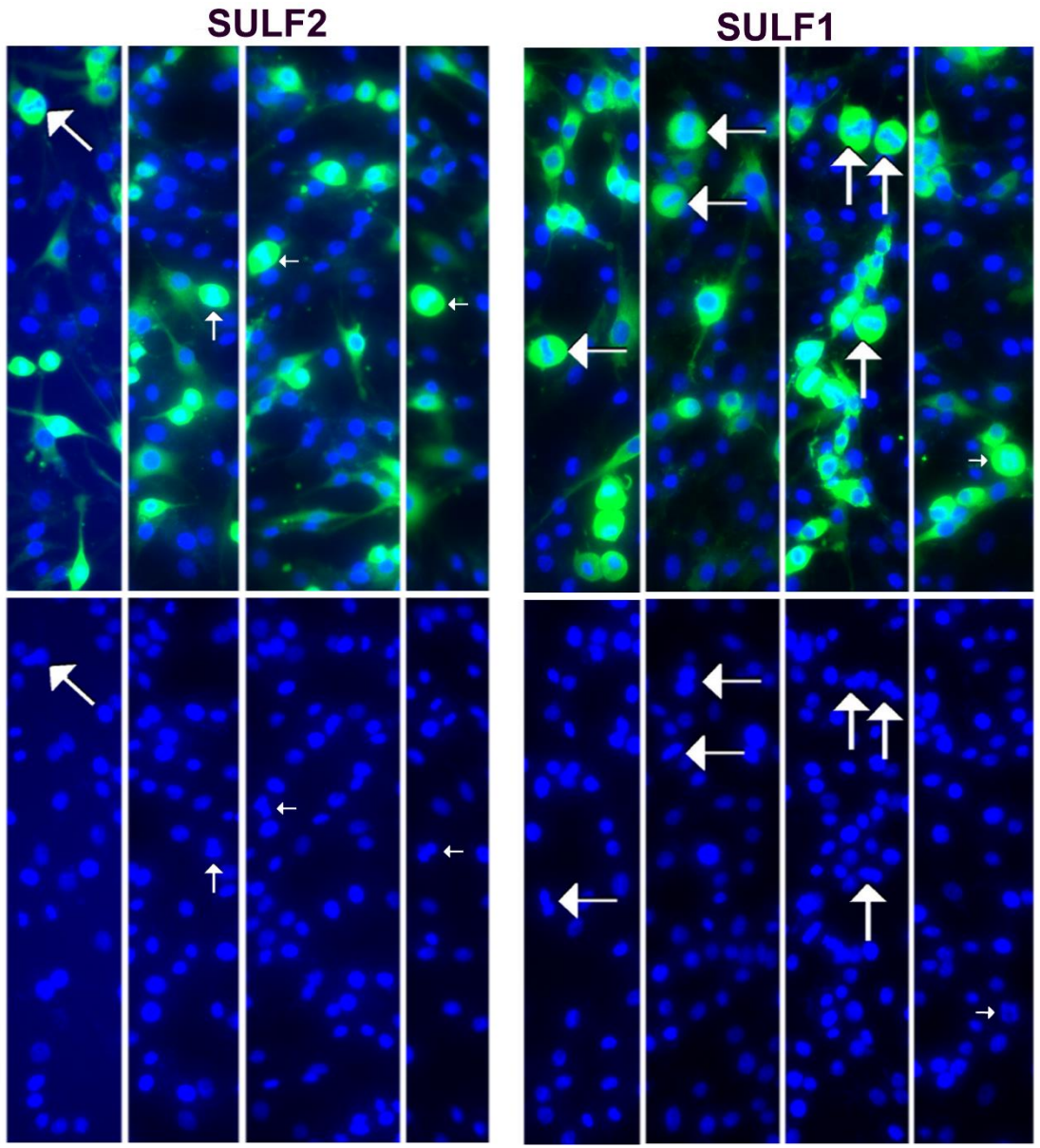
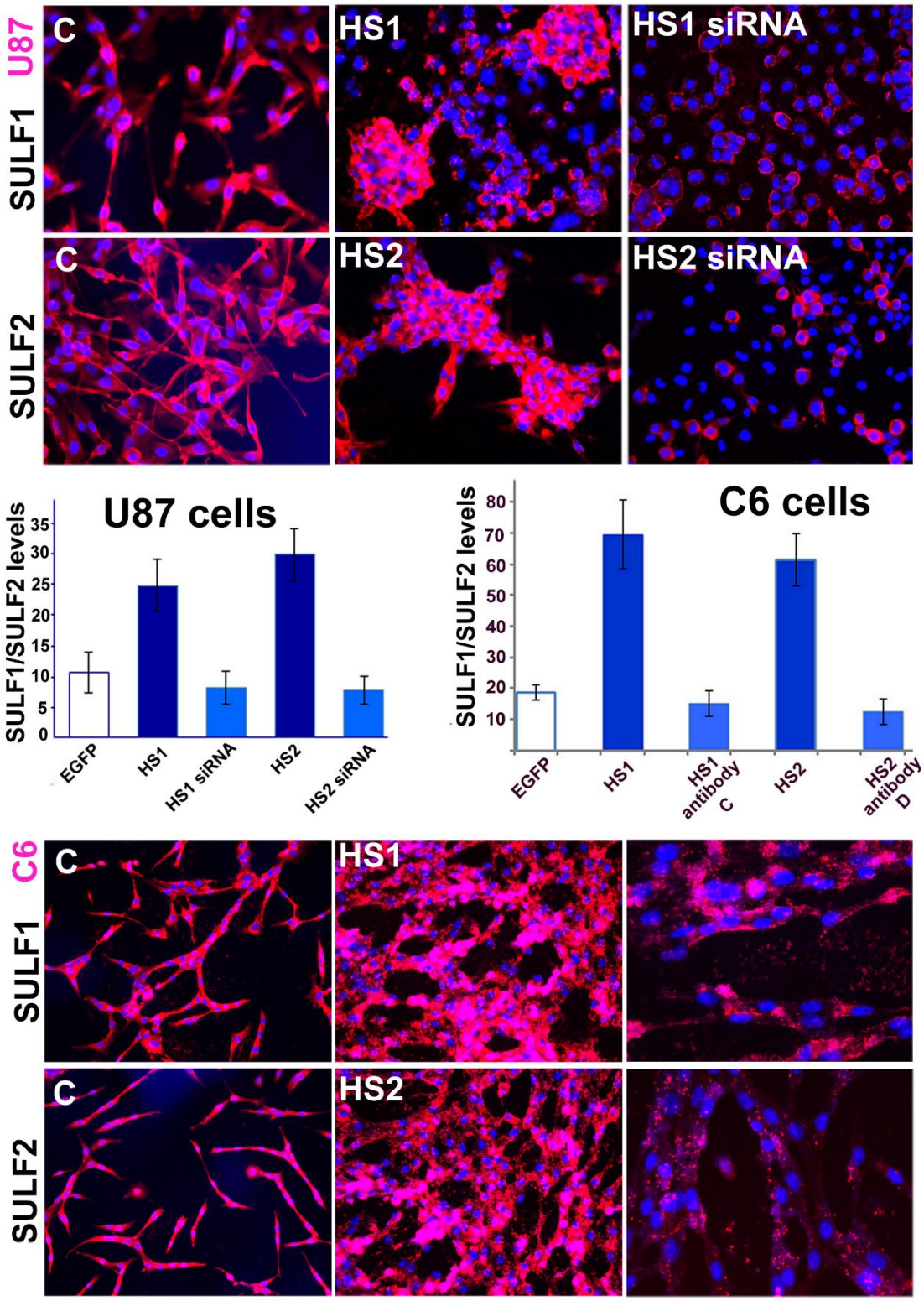
24hr
Hours after release


SULF2
SULF1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C
HS1
HS1 siRNA
SULF2
C
HS2
HS2 siRNA
U87 cells
SULF1/SULF2 levels
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30
25
20
15
10
5
0
EGFP
HS1
HS1 siRNA
HS2
HS2 siRNA
C6 cells
SULF1/SULF2 levels
80
70
60
50
40
30
20
10
EGFP
HS1
HS1 antibody C
HS2
HS2 antibody D
C6
SULF1
C
HS1
SULF2
C
HS2