

MECHANICAL AND EPIGENETIC REGULATION OF CELL PLASTICITY AND
TUMORIGENICITY

BY

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THESIS

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ABSTRACT

Nupr1 is a protein initially identified as a cancer cell marker, though its role in cancer cell metastasis and tumorigenesis is currently ambiguous. Here we identify *Nupr1* as a mechanosensitive protein involved in the regulation of tumor cell plasticity and *Sox2* expression. We monitored *Nupr1* and *Sox2* expression over time in cells cultured in soft 3D fibrin matrices. Using both untreated and *Nupr1*-siRNA treated cells, we find that high *Nupr1* expression prevents increase in *Sox2* levels. Further, we find that *Nupr1* expression is sensitive to substrate stiffness and dependent on ligand type, as *Nupr1* expression increased with substrate stiffness on polyacrylamide substrates coated with col-1 but not fibrinogen. Finally, we find that *Nupr1* is sensitive to stiffness in 3D culture as well, and is downstream of *Cdc42*-mediated cytoskeletal softening. Together, our data shows that *Nupr1* responds to the physical properties of its surroundings and can regulate tumor cell plasticity via delay of *Sox2* expression in 3D.

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I dedicate this thesis to my family, and all the friends I have made during my time in
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CHAPTER 1 || GENERAL INTRODUCTION

The process by which cells integrate mechanical signals and forces into biochemical changes, including processes such as gene upregulation, protein assembly, and transport of molecules, is known as mechanotransduction. Though soluble factors and molecular signaling have long been studied for their effects on cell behavior, much more emphasis is now being placed on a cell's mechanical microenvironment, cytoskeleton, and the cell's response to individual mechanical forces. Indeed, mechanics have been shown to play a large role in many cellular functions and behaviors^{1,2,3,4}. In particular, cell plasticity has been shown to respond to the physical properties of the cell's surroundings and exogenous forces^{5,6,7,8}.

During development of an organism, the fertilized zygote is a totipotent stem cell: that is, it can give rise to every single cell necessary for the development of the organism. As time goes on such stem cells, which also display self-renewing characteristics, divide into more specified lineages, from which they can give rise to fully differentiated cells of a specific type. This ability to self-renew and change cell type is referred to as plasticity. Beyond development, cell plasticity is also important through the adult life of an organism, as adult stem cells continually give rise to new cells within the body whenever necessary. The division, differentiation, and spatial organization/integration of such cells requires coordination of multiple signals and numerous changes in gene expression, though exactly how this occurs remains largely elusive.

Tissue engineering has long sought after the ability to control differentiation and growth of plastic cells into functional tissues and organs of choice. This ability remains unachieved, yet multiple regulating factors of plasticity have been identified and studied: particularly, the mechanical properties of the cell and its surroundings have emerged as important factors in cell fate decisions. Surface topography, substrate stiffness, and exogenous forces applied to the cell are all external factors shown to greatly affect differentiation and self-renewal; colony size, cell geometry, and cell softness are intrinsic factors which play similarly important roles as well¹².

Plasticity in Cancer

Though once thought to be an agglomeration of genetically mutated but identical cells, the tumor is now better understood as a heterogeneous, somewhat dysregulated growth of cancerous cells. The ability of a small fraction of these cancer cells to escape the primary tumor, survive in the bloodstream, and generate tumors in distant organs gave rise to the idea of cancer stem cells (CSCs)^{13,14,15}. This idea initially received traction with identification of CSC markers, but isolating these rare cells has made their study difficult, and among those that have studied CSCs exists significant disagreement, leaving a cloud of ambiguity surrounding their relevance^{10,11}; even among cancer cells displaying tumorigenicity and self-renewal, the expression of CSC markers is not consistent¹⁶.

Previously, our lab has seen that substrate softness can regulate self-renewal and differentiation in mouse embryonic stem cells (ESCs) independent of soluble factors, such as Leukemia Inhibitory Factor (LIF)⁷. Softer substrates were shown to maintain self-renewal of ESCs, while stiffer substrates caused ESCs to differentiate¹⁷. Hypothesizing that the

mechanical properties of a cancer cells surroundings may be able to affect their plastic state, we cultured B16 mouse melanoma cells in soft 3D fibrin gels of 90 Pa stiffness for 5 days*¹. Hypothesizing that the soft matrix culture may have acquired tumorigenic properties, they were injected subcutaneously into mice. Remarkably, compared to B16 cells grown on rigid plastic dishes, these soft-matrix cultured cells were up to a thousand times more likely to form metastatic tumors in the mouse lung¹⁸. These cells, dubbed tumor repopulating cells (TRCs), also exhibit high levels of stem cell genes including *Sox2*.

Mechanisms regulating plasticity in TRCs

Our most recent work has elucidated the mechanisms involved in regulation of TRC plasticity. Since TRCs exhibit changes in mechanical properties and stem cell gene expression (i.e. *Sox2*), the study focused on the relationship among cell softening, epigenetic modifications, and gene expression.

Of the numerous epigenetic mechanisms, modification of lysine tails on histone proteins is one of the foremost ways by which the cell can turn certain genes on or off. Using a FRET biosensor to monitor histone-3-lysine-9 (H3K9) di- and trimethylation (me2 and me3), we found that TRCs, unlike B16 cells grown on plastic, did not increase H3K9me when subjected to exogenous forces. The cytoskeletal softening of TRCs, which we subsequently found to be mediated by *Cdc42* expression, prevented transfer of mechanical signals to the nucleus. Importantly, H3K9me was inversely correlated with *Sox2* expression. Taken

* Recent work has shown that while the outer periphery of the primary tumor is stiffer due to presence of extra stroma and matrix proteins, the center of the tumor remains much softer. Additionally, the stiffness of metastatic colonies in a mouse lung matches the softness of the inner core of the primary tumor [18]

together, we showed that *Cdc42*-mediated softening of TRCs caused H3K9 demethylation, leading to increased *Sox2* levels and TRC self-renewal¹⁹.

Nupr1 as a mechanosensitive gene

The presented work is aimed at understanding the role and mechanosensitivity of *Nupr1*. There is much speculation and evidence for the ability of mechanical forces at the cell surface to directly cause changes in gene expression²⁰. Work has been done using artificially inserted genes, but there is need for the study of functional mechanosensitive genes.

This work shows *Nupr1* to be a mechanosensitive protein important in the regulation of tumor cell plasticity.

CHAPTER 2 || *NUPRI* AS A MECHANOSENSITIVE REGULATOR OF TUMOR CELL PLASTICITY

2.1 Introduction

Previous work has in general shown the importance of mechanics in plasticity, both in developmental and cancerous cells. There are multiple proteins and structures directly linking the nuclear membrane and periphery to the cell member, allowing transmission of force directly to genetic information. However, current studies of such events are limited by insertion of foreign genetic material. To study the effects of force-induced chromatin stretching and gene expression in live cells, it is prudent to examine a functional gene of interest. In this chapter we explore nuclear protein 1 (Nupr1) as a candidate for such a study, due to its apparent role in tumorigenicity of cancer cells.

2.2 Motivation

Nupr1 is a DNA-binding protein implicated in regulation of the cell cycle and apoptosis²¹. It was first identified as *COM1* (candidate of metastasis 1) due to its discovery as a highly upregulated marker of metastatic cancer cells²². Since its first identification, however, not much is understood of its functions in either normal or cancerous cells. Several studies have identified expression of *Nupr1* as an important player in various types of cancer cells' ability to survive^{23,24,25,26}, while others have highlighted its tumor-suppressing characteristics²⁷.

Previously, we developed a method for selecting a highly tumorigenic subpopulation of cancer cells by culturing them in soft, 90 Pa 3D fibrin matrices. This gives us an interesting platform to compare the activity of *Nupr1* in tumorigenic vs more benign cancer cells, elucidating previously unknown pathways regulating cancer cell metastasis and survival.

To further determine whether *Nupr1* will be a proper candidate for studying chromatin-stretch induced gene expression, we must first understand the response of *Nupr1* to mechanical perturbation. The proceeding sections aim to understand its mechanosensitive behaviors.

2.3 Results

2.3.1 Nupr1 delays Sox2 expression

Sox2 has previously been shown to be crucial to the self-renewing characteristics of TRCs²⁸, so first we examined the relationship between *Nupr1* and *Sox2* by looking at their expression over time. Untreated B16 mouse melanoma cells were cultured on plastic, then transferred to 3D fibrin matrices of 90 Pa stiffness for up to 5 days, with cells extracted after 1, 2, 3, and 5 days of 3D culture. RT-qPCR was used to monitor *Nupr1* and *Sox2* expression levels. In untreated cells, *Sox2* expression was found to increase after 3 days of 3D fibrin culture; interestingly, *Nupr1* levels decreased by day 2 of 3D culture, just before *Sox2* increase (Fig. 1). Seeing this behavior, we hypothesized that high *Nupr1* levels may prevent *Sox2* expression.

To test the dependence of *Sox2* expression on *Nupr1*, we repeated the above procedure with cells treated with *Nupr1* siRNA (knockdown efficiency >90%, see Fig. 1b). Simply knocking down *Nupr1* did not increase *Sox2* expression in cells cultured on rigid plastic. However, when siRNA treated cells were transferred to 3D, *Sox2* expression increased ~600% within 1 day (opposed to 3 days in untreated cells). It seems that low *Nupr1* expression allows *Sox2* levels to increase in soft 3D fibrin culture. Our data shows, however, that low *Nupr1* is not

sufficient for *Sox2* increase, due to lack of *Sox2* expression in siRNA treated cells grown on rigid plastic. Taken together, *Nupr1* appears to delay the expression of *Sox2*.

2.3.2 *Nupr1* displays ligand dependence in response to substrate properties

In 90Pa fibrin culture, cells knockdown *Nupr1* levels by ~90% (Fig. 1B). Since culturing cells in 90Pa fibrin requires a transfer of cells from a stiff, rigid plastic dish into a softer mechanical environment, we hypothesized that *Nupr1* levels may respond to substrate stiffness. To test this hypothesis we transferred cells to 2D polyacrylamide (PA) substrates of 0.15, 2, or 8 kPa stiffness for 3 hours. Additionally, we wanted to test whether any observed mechanosensitivity of *Nupr1* was due specifically to the ligands which the cell senses in its mechanical environment. The cell accomplishes this through surface receptors called integrins which display specificity of binding with various ECM ligands. To test ligand dependence, we coated the PA substrates with either collagen-1 (200 µg/ml) or fibrinogen (100 µg/ml) ECM proteins. Afterwards RT-qPCR was used to monitor changes in *Nupr1* expression. When plated on fibrinogen coated PA substrates, the cells did not change their *Nupr1* expression ($p > 0.50$) (Fig. 2A). On collagen-1 coated PA gels, however, *Nupr1* expression increased with substrate stiffness (Fig. 2B). Going from 0.15 and 2 kPa stiffness gels, *Nupr1* levels increased by ~10%, and between 2 and 8 kPa by ~20%.

As the previous set of experiments were carried out in 2D, we wanted to further explore *Nupr1*'s mechanosensitivity by perturbing stiffness in a 3D environment. To do this we compared expression levels between cells cultured in 90 or 1050 Pa stiffness 3D fibrin matrices. After 5 days culture time in 3D, cells from the 1050 Pa fibrin matrices displayed ~25% higher *Nupr1* levels compared to those from 90 Pa (Fig. 3).

2.3.3 *Nupr1* is downstream of *Cdc42*-mediated cell softening

Since *Cdc42* was previously shown to mediate cytoskeletal stiffness, and thus eventually *Sox2* expression, we wanted to see whether it also affects *Nupr1* expression levels. We have already seen that softer substrates, which lead to lower endogenous forces from the cell, lead to lower *Nupr1* expression. So, we hypothesized that knocking down *Cdc42*, which will lead to cytoskeletal softening, should also decrease *Nupr1* expression. Indeed, culturing cells on rigid plastic and treating with *Cdc42* siRNA (48hr incubation time) led to ~70% decrease in *Nupr1* levels (Fig. 4), confirming that *Nupr1* expression is downstream of cytoskeletal softening.

2.4 Conclusion

Nupr1 is an intriguing protein due to both its implication in cancer survival and its physical association with DNA. Not much is understood regarding how *Nupr1* functions in cancer. Here we show that *Nupr1* expression is correlated positively with cytoskeletal tension, displaying ligand dependent sensitivity to substrate stiffness. Further, expression of *Nupr1* delays the expression of *Sox2* in 3D soft fibrin matrices, affecting cell tumorigenicity.

It appears that *Nupr1* is most likely an intermediate between cytoskeletal softening and *Sox2* upregulation. There has been recent evidence for binding activity between MSL1 and *Nupr1* proteins in *Drosophila*, which together may be regulating histone acetyltransferase activity. It

would be interesting to see if other epigenetic changes are occurring due to Nupr1 activity, and if the *Nupr1* gene is directly regulated by mechanical force.

FIGURES

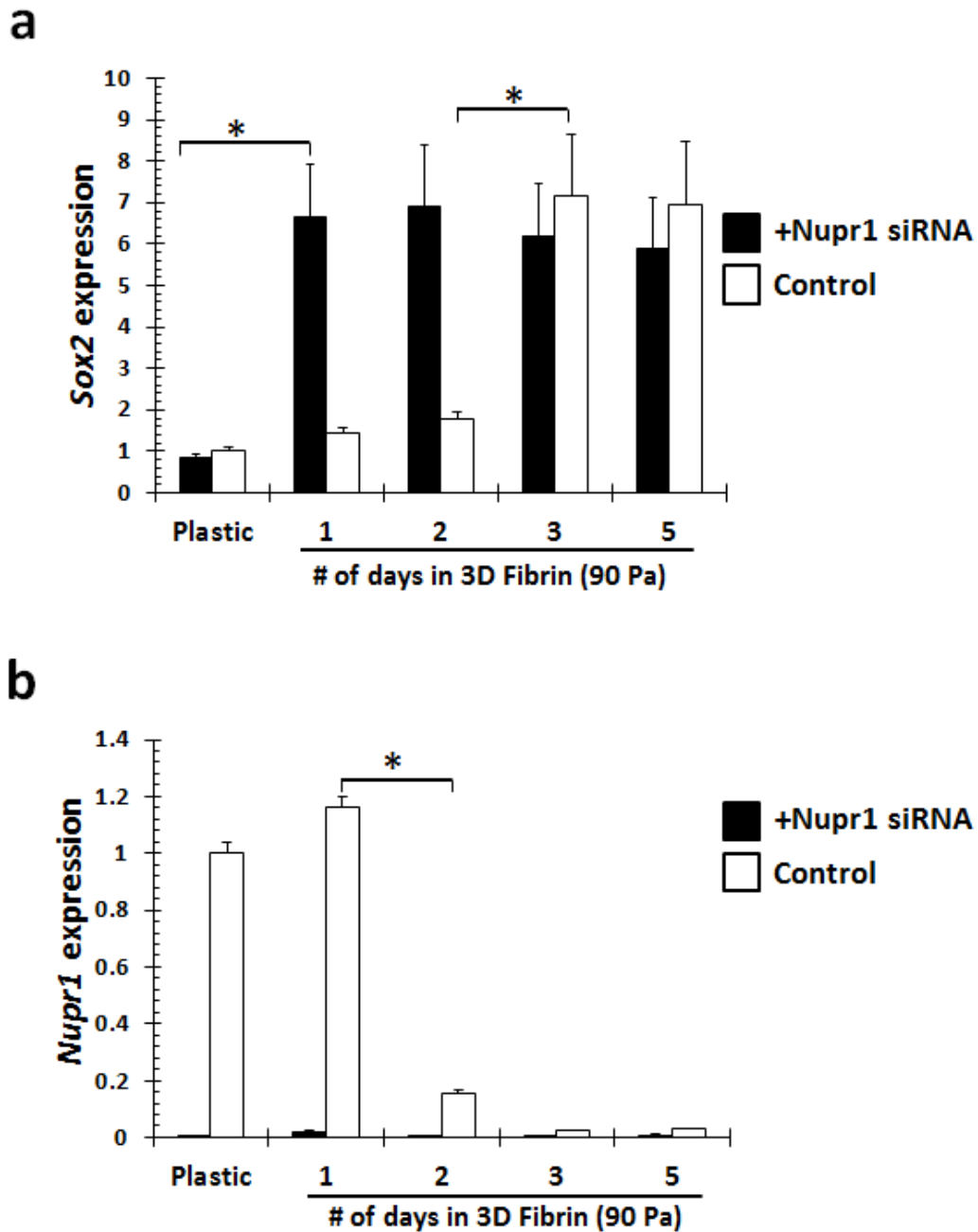


Figure 1. *Nupr1* delays expression of *Sox2* in TRCs. Cells were first cultured on plastic, then transferred to 3D fibrin culture (90Pa stiffness) for multiple days. Cells were extracted at 1, 2, 3, and 5 days. Expression of (a) *Nupr1* and (b) *Sox2* were evaluated using RT-qPCR. Mean±s.e.m. n=3 independent experiments. In untreated cells, *Nupr1* levels decrease after 2 days of 3D fibrin culture, allowing *Sox2* levels to increase soon after on day 3. In +*Nupr1* siRNA treated cells *Nupr1* levels are low, allowing *Sox2* levels to increase after just 1 day of 3D fibrin culture. *(p<0.05)

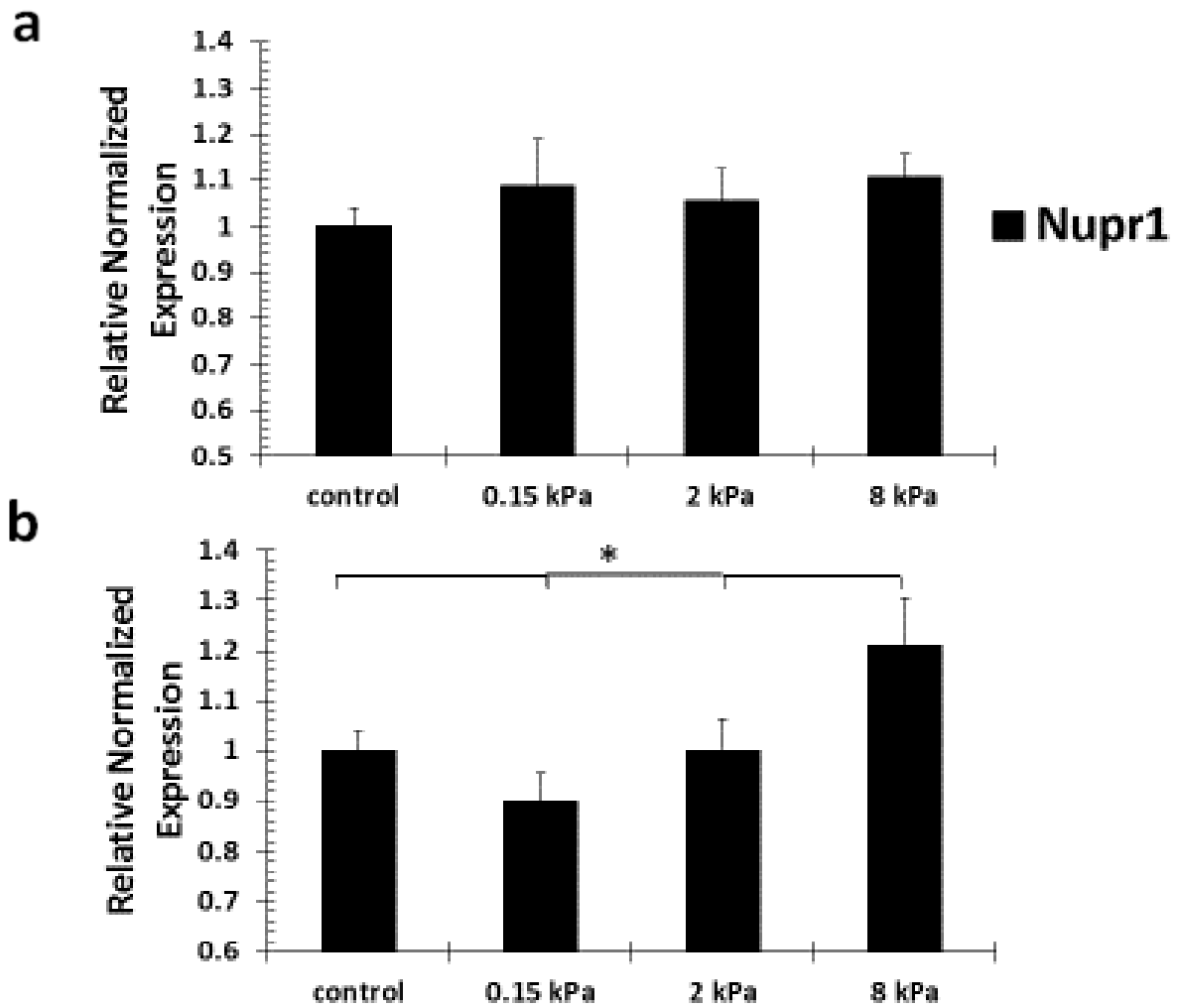


Figure 2. *Nupr1* expression increases with substrate stiffness on 2D col-1 but not fibrinogen. Cells were plated on 2D polyacrylamide (PA) substrates for 3hr. Control cells were cultured on 0.1% gelatin coated rigid plastic dishes. PA substrates were coated with either (a) fibrinogen (100 μ g/ml) or (b) col-1 (200 μ g/ml). Gene expression change was monitored using RT-qPCR, with n=3 independent experiments. Cells plated on 2D fibrinogen displayed no changes in *Nupr1* levels with changing substrate stiffness. However, cells plated on 2D col-1 increased *Nupr1* levels with increasing substrate stiffness. (Mean \pm s.e.m.) *(p<0.05)

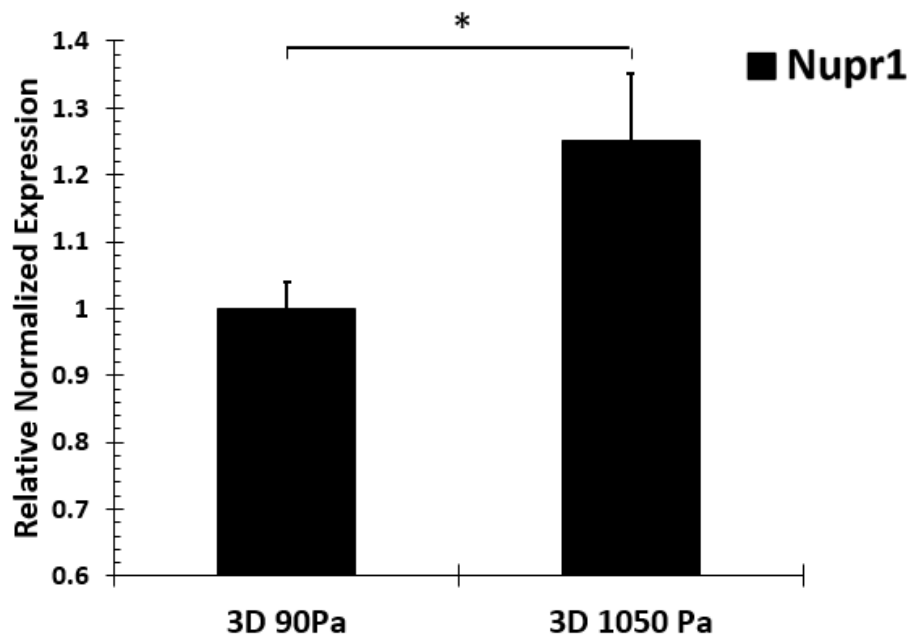


Figure 3. *Nupr1* levels increase with increasing matrix stiffness in 3D. Cells were cultured in 3D fibrin matrices of either 90 or 1050 Pa stiffness for 5 days. *Nupr1* expression was monitored using RT-qPCR with n=3 independent experiments. Increasing matrix stiffness from 90 to 1050 Pa increased *Nupr1* expression by ~25%. (Mean±s.e.m.) *(p<0.05)

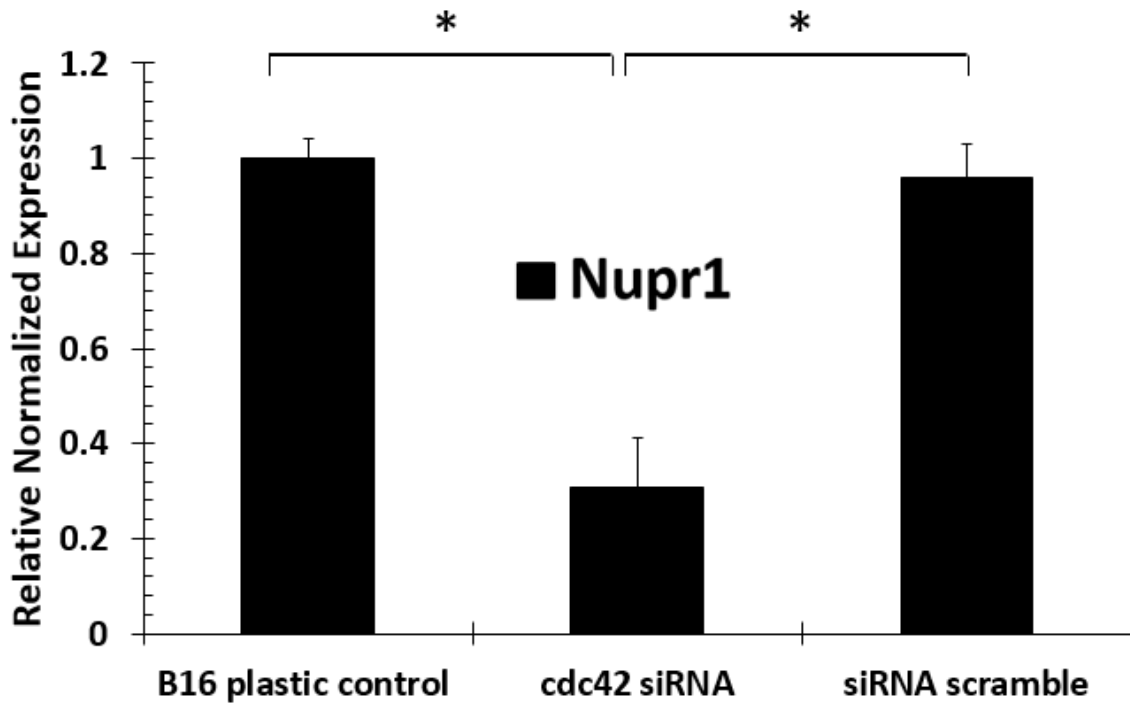


Figure 4. *Cdc42* is an upstream effector of *Nupr1*. Cells were treated with *Cdc42* siRNA for 48 hours on rigid plastic dish. *Nupr1* expression was quantified using RT-qPCR (Mean ± s.e.m.) with n=3 independent experiments. Knockdown of *Cdc42* resulted in ~70% decrease in *Nupr1* expression. *(p<0.05)

2.5 References

1. Chen, C.S., Mrksich, M., Huang, S., Whitesides, G.M., Ingber, D.E., Geometric control of cell life and death. *Science* 276:5317 1425-1428 (1997).
2. Discher D.E., Janmey P., Wang Y.L.. Tissue cells feel and respond to the stiffness of their substrate. *Science*. 310:1139–1143 (2005).
3. Krieg, M., Arboleda-Estudillo, Y., Puech, P.-H., Kafer, J., Graner, F., Muller, D.J., Heisenberg, C.-P., Tensile forces govern germ-layer organization in zebrafish. *Nature Cell Biology* 10:429-436 (2008).
4. Rauzi M., Verant P., Lecuit T., Lenne P.F. Nature and anisotropy of cortical forces orienting Drosophila tissue morphogenesis. *Nature Cell Biology* 10:1401–1410 (2008)
5. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell*. 2006;126:677–689
6. Engler AJ, Griffin MA, Sen S, Bonnemann CG, Sweeney HL, Discher DE. Myotubes differentiate optimally on substrates with tissue-like stiffness: pathological implications for soft or stiff microenvironments. *J. Cell. Biol.* 2004;166:877–887.
7. Chowdhury, F., Na, S., Li, D., Poh, Y.-C., Tanaka, T.S., Wang, F., Wang, N., Material properties of the cell dictate stress-induced spreading and differentiation in embryonic stem cells. *Nature Materials*. 9:82-83 (2010)
8. Uda, Y., Poh, Y.-C., Chowdhury, F., Wu, D.C., Tanaka, T.S., Sato, M., Wang, N., Force via integrins but not E-cadherin decreases Oct3/4 expression in embryonic stem cells. *Biochemical Biophysical Research Communications* 2:396-400 (2011).
9. Tam, W.L., Weinberg, R.A., The epigenetics of epithelial-mesenchymal plasticity in cancer. *Nature Medicine* 11:1438-1449 (2013).
10. Gupta, P.B., Chaffer, C.L., Weinberg, R.A., Cancer stem cells: mirage or reality? *Nature Medicine* 9:1010-1012 (2009)
11. Shipitsin, M., Polyak, K., The cancer stem cell hypothesis: in search of definitions, markers, and relevance. *Pathobiology in Focus* 88:459-463 (2008)
12. Li, D., Zhou, J., Chowdhury, F., Cheng, J., Wang, N., Wang, F. Role of mechanical factors in fate decisions of stem cells. *Regenerative Medicine* 6:2:229-240 (2011)
13. Bonnet, D. & Dick, J. E. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Medicine* 3:730–737 (1997)
14. Schmidt, P., Kopecky, C., Hombach, A., Zigrino, P., Mauch, C., Abken, H. Eradication of melanomas by targeted elimination of a minor subset of tumour cells. *Proceedings of the National Academy of Sciences of the United States of America*. USA 108:2474–2479 (2011).
15. Civenni, G., Walter, A., Kobert, N., Mihic-Probst, D., Zipser, M., Belloni, B., Seifert, B., Moch, H., Dummer, R., van den Broek, M., Sommer, L. Human CD271-positive melanoma stem cells associated with metastasis establish tumour heterogeneity and long-term growth. *Cancer Research* 71:3098–3109 (2011).
16. Dieter, S.M., Ball, C.R., Hoffmann, C.M., Nowrouzi, A., Herbst, F., Zavidj, O., Abel, U., Arens, A., Weichert, W., Brand, K., Koch, M., Weitz, J., Schmidt, M., von Kalle, C., Glimm, H., Distinct types of tumour-initiating cells form human colon cancer tumours and metastases. *Cell Stem Cell* 9:357–365 (2011).
17. Paszek, M. J. *et al.* Tensional homeostasis and the malignant phenotype. *Cancer Cell* 8, 241–254 (2005).
18. Plodinec, M., Lopic, M., Monnier, C.A., Obermann, E.C., Zanetti-Dallenbach, R., Oertle, P., Hyotyla, J.T., Aebi, U., Bentires-Alj, M., Lim, R.Y.H., Schoenenberger, C.-A. The nanomechanical signature of breast cancer. *Nature Nanotechnology* 7:757-765 (2012).
19. Tan, Y., Tajik, A., Chen, J., Jia, Q., Chowdhury, F., Wang, L., Chen, J., Zhang, S., Hong, Y., Yi, H., Wu, D.C., Zhang, Y., Wei, F., P, Y.-C., Seong, J., Singh, R., Lin, L.-J., Doganay, S., Li, Y., Jia, H., Ha, Y., Wang, Y., Huang, B., Wang, N. Matrix softness regulates plasticity of tumour-repopulating cells via H3K9 demethylation and *Sox2* expression. *Nature Communications* 5:4619 (2014)

20. Wang, N., Tytell, J.D., Ingber, D.E. Mechanotransduction at a distance: mechanically coupling the extracellular matrix with the nucleus. *Nature Reviews: Molecular Cell Biology* 10:75-82 (2009).
21. Chowdhury, U.R., Samant, R.S., Fodstad, O., Shevde, L.A., Emerging role of nuclear protein 1 (Nupr1) in cancer biology. *Cancer Metastasis Reviews* 28:225-232 (2009).
22. Ree, A.H., Tvermyr, M., Engebraaten, O., Expression of a Novel Factor in Human Breast Cancer Cells with Metastatic Potential. *Cancer Research* 59:4675-4680 (1999).
23. Vasseur, S., Iovanna, J.L., Le gene p8 est necessaire au developpement tumoral. *Medecine/Sciences* 19:1259-1264 (2003)
24. Brannon, K.M., Million Passe, C.M., White, C.R., Bade, N.A., King, M.W., Quirk, C.C., Expression of the high mobility group A family member p8 is essential to maintaining tumorigenic potential by promoting cell cycle dysregulation in L β T2 cells. *Cancer Letters* 254:146155 (2007)
25. Clark, D.W., Mitra, A., Fillmore, R.A., Jiang, W.G., Samant, R.S., Fodstad, O., Shevda, L.A., Nupr1 interacts with p53, transcriptionally regulates p21 and rescues breast epithelial cells from doxorubicin-induced genotoxic stress. *Current Cancer Drug Targets* 8:421-430 (2008)
26. Hamidi, T., Cano, C.E., Grasso, D., Maria Noe Garcia, Garcia, M.N., Sandi, M.J., Calvo, E.L., Dagorn, J.-C., Lomberk, G., Goruppi, S., Urrutia, R., Carracedo, A., Velasco, G., Iovanna, J.L., Nupr1 works against the metabolic stress-induced autophagy-associated cell death in pancreatic cancer cells. *Autophagy* 9:1:95-97 (2013).
27. Jiang, W.G., Davies, G., Martin, T.A., Kynaston, H., Mason, M.D., Fodstad, O., Com-1/p8 acts as a putative tumour suppressor in prostate cancer. *International Journal of Molecular Medicine* 18:981-986 (2006).
28. Liu, J., Tan, Y., Zhang, H., Zhang, Y., Xu, P., Chen, J., Poh, Y.-C., Tang K., Wang, N., Huang, B., Soft fibrin gels promote selection and growth of tumorigenic cells. *Nature materials* 11:734-741 (2012)
29. Aguado-Llera, D., Hamidi, T., Domenech, R., Pantoja-Uceda, D., Gironella, M., Santoro, J., Velazquez-Campoy, A., Neira, J.L., Iovanna, J.L. Deciphering the binding between Nupr1 and MSL1 and their DNA repairing activity. *PLOS One* 8:10:e78101 (2013)