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High Content Screening of Feeder-free Human Embryonic Stem Cells to Identify Pro-Survival Small Molecules

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Short Title: High Content Screen for Pro-survival Small Molecules

Keywords: Rho-activated kinase, ROCK, protein kinase inhibitors, pinacidil, survival, human embryonic stem cells, high content screening, drug discovery

Abbreviations: human embryonic stem, hES; ROCK1,2, Rho-associated coiled-coil containing protein kinase 1,2; PRK2, PKC-related Protein Kinase 2; FF, Feeder-free; MEF-CM, mouse embryonic fibroblast conditioned medium; TLDA, Taqman Low Density Array.

Synopsis:

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The propensity of human embryonic stem cells to die upon enzymatic disaggregation or low-density plating is an obstacle to their isolation and routine use in drug discovery and basic research. Equally, the very low rate of establishment of implanted cells hinders cell therapy. Here we have developed a high-content assay for human embryonic stem cell survival and used this to screen a range of libraries of 'lead-like' small molecules and known bioactives. From this we identified 18 confirmed hits with 4 structural classes represented by multiple compounds: a series of 5-(acyl/alkyl-amino)indazoles, compounds with a 4-(acylamino)pyridine core, simple N^6,N^6-dialkyladenines and compounds with a 5-(acylamino)indolinone core. *In vitro* kinase profiling indicated that the ROCK/PRK2 protein kinases are of pivotal importance for cell survival and identified previously unreported compound classes with this important biological activity. Evaluation using an extensive panel of protein kinases showed that six of our hit compounds exhibited better selectivity for ROCK inhibition than the routinely used commercially available ROCK inhibitor, Y-27632. In this screen we also identified the K^+-ATP channel opener, pinacidil, and show that it most likely promotes cell survival, by 'off-target' inhibition of ROCK/PRK2. We have identified novel pro-survival compounds of greater specificity, equivalent potency and reduced toxicity relative to the routinely employed ROCK inhibitor, Y-27632.

Introduction:

Poor recovery after single-cell enzymatic passage of human embryonic stem (hES) cells is a considerable obstacle to accurate cell counting and efficient culture expansion. This is of particular importance when using hES cells for drug discovery, where accurate control over cell number is critical, with evenly-distributed cells more amenable to high content screening. Recently, use of the small-molecule inhibitor Y-27632 has revealed a role for the Rho-associated coiled-coil kinases (ROCK1/2) in preventing the severe loss of viability upon cell-cell dissociation [1]. Subsequent studies demonstrated Y-27632's utility in enhancing the survival of cryopreserved hES cells [2, 3]. The mechanism by which ROCK inhibition promotes survival is still not fully understood, but may occur via inhibition of apoptosis [4]. Rho-activated kinases have been targeted by the pharmaceutical industry for their important role in vascular smooth muscle contraction and inhibitors such as fasudil are used to treat cerebral vasospasm and in ischaemic preconditioning (reviewed by [5]). The most commonly employed tool compounds for ROCK1/2, such as Y-27632 and HA-1077 (fasudil), are only moderately selective [6]. The related Rho-activated kinase, PRK2, appears to share some common functions and is also targeted by these inhibitors [7].

In order to identify compounds promoting hES cell survival with higher efficacy, improved specificity and lower toxicity, we developed a 96-well format high-content screening assay. We screened the Prestwick library of known pharmacologically active agents (1200 compounds), a focussed library of lead-like small molecule scaffolds targeted for binding in the ATP site of protein kinases (4100 compounds) and a lead-like diversity set (15,000 compounds). In this paper we describe the identification and initial characterisation of pro-survival hit compounds and their *in vitro* kinase

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inhibition profiles using a panel of >70 protein kinases. Despite screening a wide range of compounds we demonstrate that the identified pro-survival compounds all target ROCK2/PRK2 kinases *in vitro*. Furthermore, we show that pinacidil, a K^+-ATP channel opener, often used for ischaemic preconditioning in organ transplantation, has hES cell pro-survival effects through a mechanism unrelated to its effects on ion channel pharmacology; again this compound typifies the actives identified through this screen by inhibiting the ROCK2/PRK2 kinases *in vitro.* Our lead molecule is effective in promoting survival in three different hES cell lines. Together our data emphasises the critical importance of this signalling pathway in human embryonic cell survival.

Experimental:

hES Cell Culture
Human ES lines SA461, SA121, SA181, SA167 and SA002 (Cellartis AB, Dundee, UK) were maintained in a feeder-free (FF) system in a fully pluripotent state for prolonged periods by passaging every 3-4 days at a density of 3-5 x 10^4 cells/cm^2 on fibronectin (Merck Chemicals Ltd) in chemically defined medium [8] mixed at 50:50 ratio with conditioned medium derived from mouse embryonic fibroblasts (MEF-CM) (or human foreskin fibroblasts HuWIL-CM if stated) and 10 $ngml^{-1}$ bFGF (Invitrogen). Karyotypically normal SA461 cells were used in the screening process and were fully pluripotent (as judged by Oct3/4 and Nanog immunofluorescence) throughout the duration of the screening campaign.

Compound Collection and Storage
Two in-house collections of lead-like molecules assembled from commercial suppliers [9] were utilised: a kinase inhibitor focussed set (4110 compounds) and a small diversity collection (15,677 compounds). We also utilised a 1200 compound Bioactives set of marketed drugs and pharmacologically active agents (Prestwick Chemicals, France). In-house compound collections were formatted into 96-well daughter sets at 3 mM in 100% DMSO and active working sets stored for up to 4 months under nitrogen gas and with low humidity at room temperature in custom-made storage pods (FluidX).

Primary Screening
Primary screening was performed in single-point with a final compound concentration of 30 μM. 96-well CellBind plates (Corning) were pre-coated with 0.1 mg/ml fibronectin (Calbiochem) diluted in Phosphate Buffered Saline (PBS+Ca^{2+}/Mg^{2+}), stored overnight at 4°C and then aspirated immediately prior to media addition. Cell plates were prepared by dispensing 50 μl of feeder-free (FF) medium +bFGF into each well using a Wellmate dispensing robot (Thermo Fisher Scientific). 1 μl of each library compound (30 μM final concentration) was dispensed to 88 individual wells (columns 1-11) per plate using a BioMek-FX robot (Beckman Coulter). Y-27632 was used as the positive control (4 wells in column 12, final concentration 10 μM) and DMSO as the negative control (4 wells in column 12 final concentration 1%). Cells were washed with PBS, dissociated for 8-12 minutes using trypLE-select (Invitrogen) and then FF medium added to arrest dissociation. After trituration

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in a 10 ml pipette clumps were removed using a 0.45 μm tube insert filter. Cells were counted using a haemocytometer or by using a Cellometer automated cell counter (Peqlab Ltd, UK). Appropriate volumes were diluted into FF medium to give 100,000 cells/ml. 5000 cells/well in a 50 μl volume was dispensed into each well of the pre-prepared 96-well plates containing the library compounds using a Wellmate robot (Thermo Fisher Scientific). Plates were incubated for 24 hours at 37°C with 5% CO_2 before processing for imaging.

High Content Imaging
Processing of cell plates was performed using a BioMek-FX robot (Beckman Coulter Inc.) incorporating an ELX405 plate washer (BioTek Instruments Inc.). Cell medium was removed using the plate washer and wells washed twice with PBS followed by fixation at 37°C with 100 μl warm 4% para-formaldehyde in PBS (prepared fresh from 40% stock solution: Sigma). After 10 min at 37°C cells were washed and permeabilised in TBSTx (Tris Buffered Saline with 0.1% Triton X-100). After 10 min at 37°C, cells were washed twice in TBS and incubated with 1 ng/ml DAPI for 5 min at RT before two TBS washes and aspiration. 50 μl anti-fade mounting medium (0.5% *p*-phenylenediamine in 20 mM Tris, pH 8.8, 90% glycerol) was added to each well. Imaging was performed with an In Cell Analyzer 1000 (GE Healthcare) using a 10x lens, 50 ms exposure and 15 fields/well.

Image Data Analysis and Processing
Image analysis was performed to count nuclei using a customised multi-target algorithm within the InCell Analyzer 1000 Workstation software (GE Healthcare), with a filter applied to remove small DAPI-bright objects (dead adherent cells). Total "live" cell counts were summed for the well and the data exported to a csv file. ActivityBase version 5.4 (ID Business Solutions, Guilford, UK) was used for numerical data processing and analysis. Screening plates were approved if a quality-threshold criterion (Z' > 0.4) was attained. The activity of a particular compound was expressed as percent activation equal to [(experimental value - mean of low control)/(mean of high control - mean of low Control)] x 100, where the high control was 10 μM Y-27632, and the low control was 1% DMSO.

Potency Analysis
10-point concentration curves (consisting of half-log serial dilutions of compound in DMSO ranging between 10 mM and 0.508 μM stocks and 100 μM and 5.08 nM final assay concentrations) were prepared in 96-well plates using a JANUS workstation (PerkinElmer Life Sciences). All curve fitting was undertaken using a 4 Parameter Logistic dose response curve (XLFit 4.2 Model 205).

Kinase Profiling and ROCK2 IC_{50} determination
Specificity profiling utilised a panel of over 70 protein kinases in the MRC National Centre for Protein Kinase Profiling Service at the University of Dundee (http://www.kinase-screen.mrc.ac.uk). Each compound was tested *in vitro,* in duplicate, at 10 μM final concentration using recombinant kinases and model substrates. Selected compounds were submitted for IC_{50} determination

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using ROCK2 protein kinase. A 10-point half-log dilution series (top concentration 100 μM) was used. Assays were performed in duplicate.

Compound Mass and Purity Analysis
Compound was made up in DMSO (Chromasolv [Sigma]: 3-10 mM) and diluted with Optima grade acetonitrile/water (50/50, v/v) with a final concentration between 15 and 50 μM. Separations were by gradient elution on a 2.1 x 50 mm 1.7 μm BEH C_{18} column on an ACQUITY UPLC™ system (Waters ® Corporation, MA, USA). Samples were eluted with a flow rate of 0.6 mL/min. Mobile phase A was acetonitrile with formic acid (0.1%), mobile phase B was MilliQ water with formic acid (0.1%). The flow was directed through a photodiode array (scanning UV between 210 – 400 nm) and then sequentially onto the mass spectrometer (Quattro Premier XE™ triple quadrupole system, Waters, Manchester, UK), using positive electrospray ionisation in full-scan mode. Compound dilutions were analysed alongside a true DMSO blank to identify the compound-related peak in both the total ion count trace and the UV output. Extraction of the associated spectrum for these peaks confirmed identification by mass of the compound to 1 d.p. UV output confirmed purity and the presence/absence of geometric isomers (where appropriate).

Proliferation Assay
SA461 cells were plated out at 5000 cells/well in fibronectin-coated 96-well plates in fully supportive medium with 10 μM Y-27632 (Calbiochem). The following day medium was changed to fully supportive medium containing compound or controls, 8 wells/condition. Proliferation was assayed at 48 hr, 72 hr, 96 hr and 120 hrs, using a Rapid Cell Proliferation Kit (Calbiochem) . Kinase Inhibitor/Prestwick library compounds were used at the concentrations shown in the figure, the diversity library hit compounds were used at 30 μM, Y-27632 was used at 10 μM. Hits from the initial screen of the Kinase Inhibitor/Prestwick sets were tested using MEF-CM. However as this is not an ideal culture system for the development of GMP-compliant or clinically acceptable compounds, the second set of hit compounds (Diversity set) were tested in medium containing only human components (HuWIL-CM).

Long Term Compound Culture
SA461 cells were cluster passaged using trypLE-select onto fibronectin-coated 12 well plates in feeder free medium. The following day medium was changed to include compounds in feeder-free medium. Medium (feeder-free medium plus compounds) was changed every 2 days and cells were cluster-passaged when they reached confluency. Control cells were maintained up to passage 5. Kinase Inhibitor/Prestwick library compounds were all used at 5 μM with the exception of DDD00066570 (2.5 μM) and tested in feeder free medium with MEF-CM medium. Diversity set hit compounds were of lower potency and therefore were used at 30 μM and tested in feeder free medium with HuWIL-CM.

RNA isolation and quantitative PCR
Total RNA was extracted using RNeasy kits (QIAGEN) and DNaseI treatment used Turbo DNA-Free (Ambion) . The absence of genomic DNA was

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confirmed by quantitative PCR (qPCR) using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Taqman primers and probe (MWG). cDNA was synthesized with up to 2 μg total RNA in 20 μl using Superscript II (Invitrogen) The micro-fluidic 384 well TaqMan Low Density Array (TLDA) system was used to analyse samples and either the Taqman® Human Stem Cell Pluripotency Array (Applied Biosystems) with primer/probe sets for 96 genes or custom-designed plates for 24 genes. Samples were run and analysed on the 7900HT real time PCR system (Applied Biosytems).

Cell line Comparison
Cellartis cell lines SA461, SA121, SA181, SA167 and SA002 was grown in feeder-free conditions and single cell suspensions prepared as described earlier, adding either Y-27632, DDD00033325 or DMSO to the cell suspensions (10 μM final concentration or 1% DMSO) and then seeded at 0.5ml/well in fibronectin-coated 24-well plates, in triplicate. After 24 h each well was washed and a single cell suspension generated by using extensive trypsinisation using trypLE-select. Cell numbers were counted using a Cellometer (Peqlab) and cells/ml calculated. Fold survival values for DDD00033325 and Y-27632 were calculated relative to the DMSO control.

Results

Development of high-content single-cell hESC survival assay
In order to identify novel small molecules that act as pro-survival agents we developed a high-content, 96-well format, screening assay. Methodology was developed to extensively disaggregate and filter hES cells to give single cells – this enabled accurate counting and plating using a bulk dispensing robot to give low variance in cell numbers between wells. Y-27632, a commonly employed Rho-kinase inhibitor that promotes stem cell survival [1] was used as a positive control compound and DMSO used as a negative control. In the presence of the reference compound (Y-27632) over 90% of cells survive and are retained as viable cells on the substratum, in contrast to the control conditions where very few cells were adherent and viable (Fig. 1A). Nuclei from non-specifically adhered dead cells were observed to a low level in the absence of the reference compound which, if counted, would reduce the screening window. However these were excluded from the measurements on the basis of nuclear size and signal intensity using the analysis software filtering tools, underlining the flexibility of using a high-content screening strategy over a plate reader-based approach. Seeding density was found to be critical since at very high densities (>20,000 cells/well) a self-supporting effect was apparent, possibly due to rapid re-aggregation or growth factor secretion (data not shown). Compound addition into medium in fibronectin-coated wells of screening plates was performed immediately prior to the addition of cells and cell processing time kept to an absolute minimum. Blinded mock screening was performed at five different concentrations of Y-27632 (0.1 μM, 1 μM, 2 μM, 5 μM and 10 μM) to test for assay performance and this showed that at concentrations above 2 μM 100% of the spiked wells were identifiable as “hits” (Fig.1B). The screens involving the Dundee compound collections (i.e. kinase and diversity sets) were performed at a final

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concentration of 30 μM, whereas the average screening concentration of bioactives library was 62.5 μM (stock concentration range of 1.2–19.59 mM).

Hit Identification and screen performance

On the basis of a promising assay window and high-quality mock screening, we undertook a proof-of-concept screen using the 4110 compound library of kinase-targeted lead-like ATP-competitive inhibitor scaffolds [9]. The performance of the screen, measured by the Z' value [10] for each plate, is shown in Fig 2A. The assay was found to be robust and achieved a signal to background of > 10:1 with an average Z' value for the screen of 0.76 ± 0.09, and a mean % coefficient of variation (% CV) for the reference compound of 5.9 ± 2.3% (Fig. 2B). Plotting a frequency distribution of percent activation (increase in viable cell count over DMSO control normalised to the difference between high control and low controls) showed the data lay within a normal distribution (Fig. 2C). The cut-off for hit definition was set at compounds returning percent activation values in excess of 3 standard deviations away from the mean of the negative control (i.e. background). On this basis and manual image inspection, all 51 putative hits were 'cherry-picked' and tested in dual point retests and subjected to mass and purity confirmation by UPLC and LC-MS/MS. Of these, 6 were deemed confirmed using 10-point concentration-response curve retests with potencies that ranged from 3 μM to > 30 μM, giving a hit rate for this screen of 0.15%. A combination of re-purchasing and re-synthesis enabled further retesting of fresh material using a higher top concentration of 100 μM and confirmation of activity and efficacy of the confirmed hits (See Fig. 2D for example curves, Fig.4 and Table S1).

We also screened the commercially available 1200-compound bioactives library of drugs and known pharmacologically-active agents (Prestwick). A single hit was identified giving 60% activation relative to the reference compound Y-27632 (this particular compound is supplied at 8 mM in the Library). The compound was found to be pinacidil, a K^+–ATP channel opener and vasodilatory drug used in the reduction of blood pressure [11]. Pinacidil was repurchased and verified in 10-point dose-response curves, giving an EC_{50} of 46 μM (Fig.4 and Table S1).

In order to extend our coverage of chemical space and perhaps identify hits with a non-kinase mode of action, a small diversity collection (15,667 compounds), a representative subset of a larger diversity set (65,000 compounds) [8], was screened. 18 compounds with a percent activation of > 3 SD over the low control were identified as putative hits and taken into dual-point retesting alongside mass/purity determinations. Of these initial putative hits, 12 compounds were verified as positive and retested for potency. Resupply material was obtained for all the confirmed 12 hits, their mass/purity confirmed, and retesting performed up to a higher top concentration of 100 μM, giving EC_{50} values ranging from 16 μM to 49 μM (Table S1).

***In vitro* kinase profiling of hit compounds**

In order to provide insight into the kinase inhibition profiles of the hits identified, compounds were subjected to panel screening by the MRC National Centre for Protein Kinase Profiling at the University of Dundee.

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Screening was performed at 10 μM in duplicate using recombinant kinases and model substrates [6]. The full results of the analyses are listed in Table S2, with examples shown in Fig. 3A for the reference compound, the apparently most selective hit (DDD00033325) and pinacidil. The striking observation derived from these data was the fact that ROCK2 and/or the closely related PRK2 were inhibited to a significant degree by all survival compounds tested (Fig 3B,C). DDD00082896 and DDD00074187 were exceptional in not significantly inhibiting PRK2. In fact, in general, the hit compounds exhibited a higher degree of inhibition against ROCK2 than PRK2 in the *in vitro* assay (Fig. 3C). Beyond this, there was little other commonality in kinases inhibited (Fig. 3B). The inhibitory activity of the compounds against ROCK1 was not defined in this study, as this kinase, which is very closely related in its kinase domain to ROCK2, was not present in the profiling panel. An IC_{50} analysis of several hits with hESC survival EC_{50} values < 20 μM was subsequently performed to compare their ROCK2-inhibitory activities with Y-27632. As expected, the *in vitro* determined IC_{50} values for the compounds tested are all 5-20 fold lower than the equivalent EC_{50} values determined phenotypically (Fig.4 and Table S1) and, although the most potent ROCK inhibitor *in vitro* is also the most potent compound in the survival assay, the data set available is not large enough to delineate a clear correlation.

Whilst a number of the compounds possessed a degree of selectivity for inhibition of ROCK2 and PRK2, most notably DDD00033325 (See Fig. 3B), many of the hits showed low ROCK2/PRK2-specificity. This is unsurprising for compounds issuing from the focussed kinase set, since all of the compounds in this library are designed to be ATP-competitive kinase inhibitors. The reference compound, Y-27632, as expected, also inhibited ROCK2/PRK2 but, additionally, inhibited several other kinases in the panel, which is consistent with previous studies showing that this purported ROCK-specific inhibitor does exert significant off-target actions on other protein kinases [6, 12] (Fig. 3 & Table S2). Most commercially available, claimed Rho kinase-specific inhibitors also show poor selectivity towards ROCK [12]. In marked contrast to this, however, six of our hit compounds (DDD00033325, DDD00033207 and DDD00082896, DDD00074187, DDD00074102, DDD00076032) showed reasonably clean profiles against non-ROCK/PRK kinases in the panel employed.

Of particular interest is the K^+–ATP channel opener pinacidil, which displayed moderately selective inhibition of ROCK2/PRK2 (Fig. 3 and Table S2). This selective, albeit nominally off-target effect is potentially of importance as Rho kinase inhibitors are, like pinacidil, used in ischaemic preconditioning. Indeed, pinacidil shares a common functionalised 4-aminopyridine core structure with both Y-27632 (Fig. 4) and other established ROCK inhibitors [13] so this previously unreported ROCK-inhibitory activity of pinacidil has a reasonable structural basis. To confirm that pinacidil's effect on hESC survival is likely due to kinase inhibition, rather than ion channel activity, the K^+–ATP channel blocker glibenclamide, known to reverse the effects of pinacidil on K^+ flux, was used. Glibenclamide had no adverse effect on hESC survival alone, nor was able to block the effect of either pinacidil or Y-27632 on survival as shown in Fig. S1. One other compound in our screen output, DDD00079566 shared

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the ROCK-inhibitory 4-(acylamino)pyridine structure (see Fig. 4). This compound and pinacidil are therefore expected to share a common binding mode with Y-27632, for which co-crystal structures with ROCK1 and ROCK2 established that the pyridyl nitrogen acts as a key hydrogen bond acceptor to engage the backbone of the enzymes' MEYMPG hinge sequence [14, 15].

Chemistry-based analysis of the full hit set revealed numerous core structures that are commensurate with ROCK-inhibitory activity. Thus, DDD00074187 is structurally related to the established ROCK inhibitor, HA-1077 (fasudil; Fig. 4), for which ROCK1 and ROCK2 co-crystal structures exist [15, 16]. In these structures the pyrido ring of the isoquinoline superimposes very closely onto the position occupied by the pyridine ring of Y-27632 in the co-crystal structures of the latter. Many established ROCK inhibitors such as fasudil and Y-27632 feature a basic nitrogen positioned 9-12 Å from the hydrogen bond acceptor of the core subunit that anchors the inhibitor to the kinase hinge. This feature allows engagement of one or other of a number of aspartic acid residues located at the rim of the ATP binding site, including the aspartic acid of the conserved kinase DFG motif. The structures of both DDD00033325 and DDD00033207 embody a basic nitrogen at a suitable distance from the N-1 centre in their respective pyrazolo[1,5-*a*]pyrimidine and imidazo[1,2-*b*]pyridazine core structures to allow adoption of such a binding mode. Interestingly, DDD00033325 is isosteric with an indazole (Iwakubo **5b**, Fig. 4) recently disclosed as a potent ROCK inhibitor [13, 17]. The proposed binding mode for indazole ROCK inhibitors of this type [17] exploits the respective hydrogen bond donor and acceptor capacities of both N-1 and N-2 centres for engagement of the kinase hinge sequence to provide strong inhibitory potency. The strong intrinsic ROCK-inhibitory activity of the 5-substituted indazole core is reflected in the identification of a series of six 5-(acylamino)- and 5-(alkylamino)indazoles in our hESC survival screen output (DDD00046328, DDD00046780, DDD00047425, DDD00074083, DDD00074102 and DDD00074270; Fig. 4).

Two other less well-populated series of hits were also identified in our screens. Firstly, a group of N^6,N^6-dialkyladenines, a class of compounds not previously reported as ROCK inhibitors, was discovered in DDD00066444, DDD00066508 and DDD00081921 (Fig.4). Secondly, a pair of related 5-(acylamino)-1-methylindolinone (or thiaindolinone) compounds was found in DDD00076032 and DDD00082896 (Fig.4). A third indolinone (DDD00066570) may be related in that the 2-oxo group of its core heterocycle may serve as a hydrogen bond acceptor feature for interaction with the kinase hinge sequence. In this latter compound the unsubstituted indolinone nitrogen may additionally act as a hydrogen bond donor to the kinase hinge in a manner analogous to that proposed for the indazoles [13, 17]. Indeed, such interactions are supported by the co-crystal structures of related indolinones, SU5402 and SU6656 (Fig. 4), with the kinase domains of CaMKII and FGF-R1 [18] [27]. DDD00066570 proved the least selective compound in our hESC screen output, showing strong inhibition of FGF-R1 and numerous other kinases in the test panel (Fig. 3B and Table S2).

Effects on Long-term Growth and Pluripotency

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In order to determine whether our pro-survival compounds had effect on cell physiology if used beyond a short-term culture, cell proliferation was monitored for up for five days at a compound concentration around the EC_{50} value in the survival assay. Two compounds (DDD00066444 and DDD00066570: Fig. 5A) strongly suppressed proliferation; the remaining hits showed only modest or no effect on cell proliferation (Fig. 5A, 6A). Interestingly we consistently observed a positive effect of pinacidil on cell proliferation. hES cells were next treated persistently with the compounds under normal growth and passaging conditions to determine how sustained exposure affected cell proliferation (Figs. 5B & 6B). As seen in the short-term proliferation assay described above, compounds DDD00066444 and DDD00066570 adversely affected cell growth, while the cells behaved as control cultures in the presence of all other compounds tested. Cells could be maintained in DDD00066444 by passaging cells at a higher density up to passage 5, however cells grown in DDD00066570 were arrested in their growth at passage 1 and high concentrations were toxic (Fig. 5B and J.G. unpublished observations). In addition cells grew particularly slowly in compound DDD00074187 and only reached passage 2, cells grown in DDD00074102, DDD00074083 and DDD00036524 reached passage 3 and in DDD00046780 passage 4.

To assess the maintenance of pluripotency in the presence of the compounds, cells were stained for Oct3/4 and also for Cytokeratin-7 as an early differentiation marker for trophectoderm. Oct3/4 staining was maintained throughout the experimental time-course in all samples (Fig. S2). Occasional small patches of differentiated cells staining positive for Cytokeratin-7 (< 5% of the total cells) were observed in all samples including untreated and Y-27632 treated cultures. This level of spontaneous differentiation is not unusual during hES cell maintenance and was not altered by the compounds.

Gene expression analysis of mRNA extracted from cells exposed to compounds was performed using Taqman low-density arrays (TLDA). Results of the analysis of the cell treated with Kinase Inhibitor/Prestwick compound sets are shown in Fig. 7A. Key pluripotency genes were mostly unaffected by the compounds – treatment with the compounds or Y-27632 resulted in less than 2.5 fold difference in gene expression when compared to the untreated control. Cells treated with DDD00066570 and DDD00066444 showed increased expression of some endoderm and mesoderm-specific genes such as eomesodermin, Sox17, brachyury, GATA4 and GATA6 indicating these compounds may be inducing differentiation in addition to reducing the proliferation of hES cells. Gene expression analysis for the diversity library hits was performed over the course of the culture at passages P1, P3 and P5. As shown in Fig. 7B, pluripotency was maintained and most compounds induced very little change in expression of the pluripotency markers Oct3/4, Sox2, Nanog and DNMT3B. There were some small changes in gene expression for several of the differentiation markers; however these likely reflect the small patches of differentiated cells observed in the stained cells. Since it is widely known that distinct hES cell lines can behave differently in culture we tested our lead compound DDD00033325 and Y-27632 against five separate cell lines (SA461, SA181. SA167, SA121 and SA002). We

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observed that DDD00033325 promoted survival and was as effective as, or more effective, than Y-27632 in all cell lines tested (Fig. 8).

Discussion:

Unlike mouse ES cells, human ES cells die if dissociated into single cells by enzymatic treatment, a property that hampers their isolation and manipulation for both drug discovery and basic research. In this study we performed a high content screening campaign using fully pluripotent human ES cells, growing in feeder-free monolayer cultures in 96 well format, in order to identify small molecules that promote single cell survival. We screened a bioactives library in addition to two libraries of lead-like small compounds – one designed for chemical diversity and the other designed to target the active site of protein kinases. We identified a number of hit series, some with structural similarity to known Rho-kinase inhibitors and others with structural novelty. Some compounds were found to be less toxic than existing Rho-kinase inhibitors and some induced differentiation in hES cells on long-term exposure. Remarkably, despite using three distinct screening libraries, the small molecules identified all inhibited ROCK2 and/or PRK2.

The high content screening assay described here was robust and gave good signal to background values using the known ROCK inhibitor Y-27632 as our reference compound [1]. High content analysis allowed the exclusion of dead cells from the analysis and demonstrated the advantages of using this platform over a plate reader-based analysis. We initially screened our 4000+ compound library of kinase inhibitor scaffolds. This screen identified 5 small molecules with pro-survival activity. Each hit was verified with resupply/resynthesised material and concentration-response curves revealed potencies ranging from 2 to 50 μM. We noted that at the highest concentration tested (100 μM) cell toxicity was observed, including for the reference compound Y-27632 (see Fig. 3). Indeed Y-27632 had optimal effect on survival at 3 μM with higher concentrations leading to > 60% reduction in cell numbers. Two compounds (DDD00033325 and DDD00066508) were efficacious up to 30 μM and only toxic at 100 μM. Several of the hit compounds showed toxicity above 30 μM that may not necessarily be related to ROCK2/PRK2 inhibition. Kinase profiling demonstrated that Y-27632 inhibited ROCK2 and PRK2 to 80% (at 10 μM final concentration in the assay) but also inhibited RSK1, MSK1, MNK2, PHK and BRSK2 to a similar degree, as previously reported [6]. This lack of specificity supports previous experiments, which showed that several kinases (including ROCK1, ROCK2 and PRK2) contribute to the global effect of Y-27632 on cellular responses [7]. The most ROCK2/PRK2-selective hit was DDD00033325, which showed strong inhibition of ROCK2/PRK2 as well as cAMP-dependent protein kinase PKA (and weakly the mitogen and stress regulated kinase, MSK1).

We also screened the Prestwick bioactives library, which includes known pharmacological agents and marketed drugs. The single low potency hit identified was the ATP-sensitive K^+-channel opener pinacidil, a drug used as a vasodilator and in ischaemic preconditioning of organs in transplantation [19]. Geron used pinacidil in its pro-survival cocktail employed to sustain transplanted cardiomyocytes derived from hES cells in a rat infarct model,

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although its inclusion was to mimic ischaemic preconditioning rather than promote cell survival *per se* [20]. Our data indicate that the pro-survival effects of pinacidil are not due to the compound's effect on K^+ channel opening. Given the structural similarity of pinacidil to established ROCK inhibitors including Y-27632 (*vide supra*), we considered that the compound's hESC survival effect was likely due to off-target kinase-inhibitory action. This was subsequently confirmed by panel profiling that revealed modest activity against ROCK2 and the related kinase, PRK2, (respectively 60% and 70% inhibition at 10 μM) but little or no activity against the other 70+ kinases tested in the panel.

To expand the coverage of chemical space and possibly uncover novel effectors of cell survival a small diversity library was screened identifying several hits. 12 compounds with low micromolar potencies were verified. Examination of the hit chemical structures revealed similarities to known ROCK inhibitors in several instances (*vide supra*), and kinase profiling confirmed that the most potently inhibited kinases were ROCK2/PRK2. This result was striking since the diversity library, unlike the kinase inhibitor library was not assembled to target kinases specifically. This result shows that a phenotypic screening approach identified specific kinase inhibitors and underlines the pivotal importance of the Rho-activated kinase pathway in the regulating the cellular response to lack of homotypic and heterotypic interactions.

Several compounds (DDD00082896, DDD00076032, DDD00074187, DDD00033325, DDD00033207) were more selective for ROCK2/PRK2 than the reference compound Y-27632, and these might be of interest to pursue to dissect the role of ROCK/PRK in cytoskeletal rearrangement. An observation worthy of further study was that three compounds (DDD00082896, DDD00076032, DDD00074187) were relatively selective for ROCK2 over PRK2 but exhibited low potency in the phenotypic assay compared to compounds such as DDD000033325, which inhibited the two enzymes to a similar extent. Further work would be required to determine their *in vitro* selectivity profiles. Since both ROCK1,2 and PRK2 are thought to act in concert, at least in cytoskeletal signalling [7], it is possible both these related kinases have a role to play in hESC survival. Of further interest is the observed inhibition of EPH-B3 receptor tyrosine kinase by both DDD00074102 and DDD00074187, since this receptor family is known to be involved in signalling in cell-cell adhesion [21]. Evidence also suggests that signalling via the FGF receptor plays a role in sustaining self-renewal partly via the cytoskeleton and in the prevention of anoikis [22]. Data from a recent screen [23] for pro-survival small molecules has shed further light on this effect with the identification of a ROCK inhibitor (thiazovivin) structurally unrelated to those described here. The study provided evidence that contraction of the cytoskeleton mediated by the Rho-ROCK pathway leads to cell death [23].

One of the concerns in the stem cell field is the potential for adverse effects of small molecules on cell growth and proliferation, and, if present in the culture media for extended periods, their effects on stem cell pluripotency. In the

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context of transplanted progenitor cells this could compromise their cellular identity and differentiation capacity. We noted that in our survival assay, which was of 24 hours duration, certain compounds, including Y-27632, showed toxicity at higher concentrations (between 10 and 30 μM). In order to address these potential issues we cultured hES cells with compounds (30 μM) from 48 hours up to 120 hours and monitored cell proliferation. A subset of the compounds suppressed growth on long-term exposure (DDD00066570, DDD00066444, DDD74102, DDD00036524, DDD74083), however the majority did not have a marked effect.

To further address issues relating to continual exposure to compounds, hES cells were grown with compounds for up to 5 passages and gene expression analysis was performed by TLDA. This showed that most compounds had little or no effect on the expression of a variety of pluripotency genes and differentiation markers. The two compounds from the kinase inhibitor library that reduced proliferation also increased expression of mesoderm or endoderm markers. DDD00066570 predominantly up-regulated endoderm markers, including GATA4, GATA6 and Sox17, but also eomesodermin. Similarly DDD00066444 increased expression of eomesodermin, brachyury, Sox17 and GATA6. Two receptor tyrosine kinase inhibitors structurally related to DDD00066570 (SU5402 and SU6656; see Fig. 4) have been shown to have effects on hESC differentiation [24-26], indicating a potential mechanism for the observed effects of DDD00066570. These data suggest that ROCK-inhibitory compounds that lack selectivity in targeting ROCK/PRK2 may be incompatible with maintaining pluripotency in culture over extended periods, but could have utility in steering differentiation to particular germ layers in some instances. Minimal exposure of hES cells to small molecule survival agents may be good practice in general to minimise risk of unwanted effects on the cells, however, and might be achieved if media change can be performed once cells are attached. The gene expression data presented here were obtained using mRNA from cells growing in feeder-free conditioned medium derived from fibroblast cultures – it may well be that differences in medium recipes and sources of conditioning medium could influence the outcome of continued exposure to compounds. More work using different culture conditions would be needed to test the effect of compound, in particular using chemically-defined proprietary media. In order to understand the general applicability of the ROCK-inhibitor effect we demonstrated that, at least for our lead compound DDD00033325 and Y-27632, five different cell lines tested were able to survive single-cell disaggregation when ROCK/PRK2 was inhibited, indicating a general efficacy towards hES cells.

In summary, we have identified novel pro-survival compounds of greater specificity, equivalent potency and reduced toxicity relative to Y-27632. Notably, our most ROCK/PRK2-selective compound (DDD00033325) promoted single cell survival up to 20-fold over background, had little or no effect on hES cells in long-term culture, was effective on more than one cell line and therefore has the potential for routine use requiring work with disaggregated hES cells. Clues as to the mode of action of these compounds in hES cells revealed a striking and consistent link to inhibition of ROCK kinases, despite identifying a range of hits with structural diversity. These data

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further underline the pivotal nature of the ROCK kinase in regulation of hESC survival as single cells. ROCK's reported role in cytoskeletal re-arrangement and adherence and recent data from small molecule inhibitor studies [23] relate well to our observations using these phenotypically identified small molecules.

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Figure Legends:

Figure 1. Survival Assay and mock screening results
(A) Assay optimisation: Image shows DAPI stained cells (one field per well) a 96 well test plate. Cells were plated at decreasing density (50,000/well to 1,000 per well) from left to right, with no cells in columns 11 and 12. The top 4 rows have 10 μM Y-27632 present and the lower 4 rows DMSO. (B) Results of blind mock screen. Histogram showing percentage activation (compared to DMSO control) in spiked wells against concentrations of Y-27632 used (n=4).

Figure 2. Survival Assay Hit Discovery and Potency (A) Z' plot for Kinase library screening (B) Performance statistics for the kinase library screen. (C) Frequency distribution curve for kinase library screen (inset shows details of those hits which were selected for further analysis) (D) Concentration-response analysis of DDD00033325 and reference compound Y-27632. Example potency curve for the top hit from the kinase library, note the toxic effects at higher concentrations of Y-27632.

Figure 3. *in vitro* kinase panel profiling of hit compounds. (A) Examples are shown of the percentage inhibition of protein kinases in the MRC Unit's kinase panel. Top panel DDD00033325, middle panel pinacidil and lower panel Y-27632. Values are mean of duplicate assays performed at 10 μM compound concentration using recombinant kinases and model substrates. (B) Heatmap representation of kinase inhibition. Red indicates maximal inhibition, green no inhibition. (C) Table summarising percentage inhibition of ROCK2 and PRK2 kinases in the presence of hit compounds (tested at 10 μM). (B) Heat map of all hit compounds against kinase panels.

Figure 4. Structures of hESC survival screen hits and relationship to established ROCK and other kinase inhibitors. DDD00079566 and pinacidil are structurally related to Y-27632 and other 4-(acylamino)pyridine ROCK inhibitors. DDD00046328, DDD00046780, DDD00047425, DDD00074083, DDD00074102 and DDD00074270 are structurally linked to indazole ROCK inhibitors exemplified by Iwakubo **5b** [17]. DDD00033325 is isosteric with Iwakubo **5b**. DDD00074187 is structurally related to the established ROCK-inhibitory isoquinoline, fasudil. DDD00066570, the least selective of the hESC survival hits, is structurally associated with the tyrosine kinase inhibitors, SU5402 and SU6656. EC_{50} values from the cellular pro-survival assay are shown for each compound. ROCK2 *in vitro* IC_{50} values are also shown for selected compounds.

Figure 5. Effects of Kinase and Prestwick library hit compounds on growth (A) Cell proliferation measured at three timepoints for each compound plus DMSO/Y-27632 controls. (B) Bright field photographs of SA461 cells cultured with compounds or controls. Cells show reduced proliferation on extended passage in the presence of DDD00066444 and DDD00066570 compounds. Scale bar represents 100 μm.

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Figure 6. Effects of diversity library hit compounds on growth (A) Cell proliferation assays measured at three time points for each compound versus positive (Y-27632) and negative (DMSO) controls. (B) Bright field photographs of SA461 cells exposed over long-term culture to hit compounds or controls. Scale bar represents 100 μm.

Figure 7. Effects of hit compounds on gene expression markers. (A) Heat-map of qPCR data for cells passaged five times in the presence of kinase/Prestwick library hits. (B) Heat-map of qPCR data for cells at passage one, three or five in the presence of diversity library hits.

Figure 8. Effect of DDD00033325 and Y-27632 on cell survival in five different cell lines. Each of the three cell lines (SA461, SA181, SA167, SA121 and SA002) were disaggregated to single cells, counted and diluted before compound or DMSO addition (10 μM final concentration) and seeding at medium density in 24 well plates in triplicate. After 24 hr incubation wells were washed and cells were trypsinised to a single cell suspension for counting. Average fold survival for each line was calculated for DDD00033325 and Y-27632 relative sto the DMSO control.

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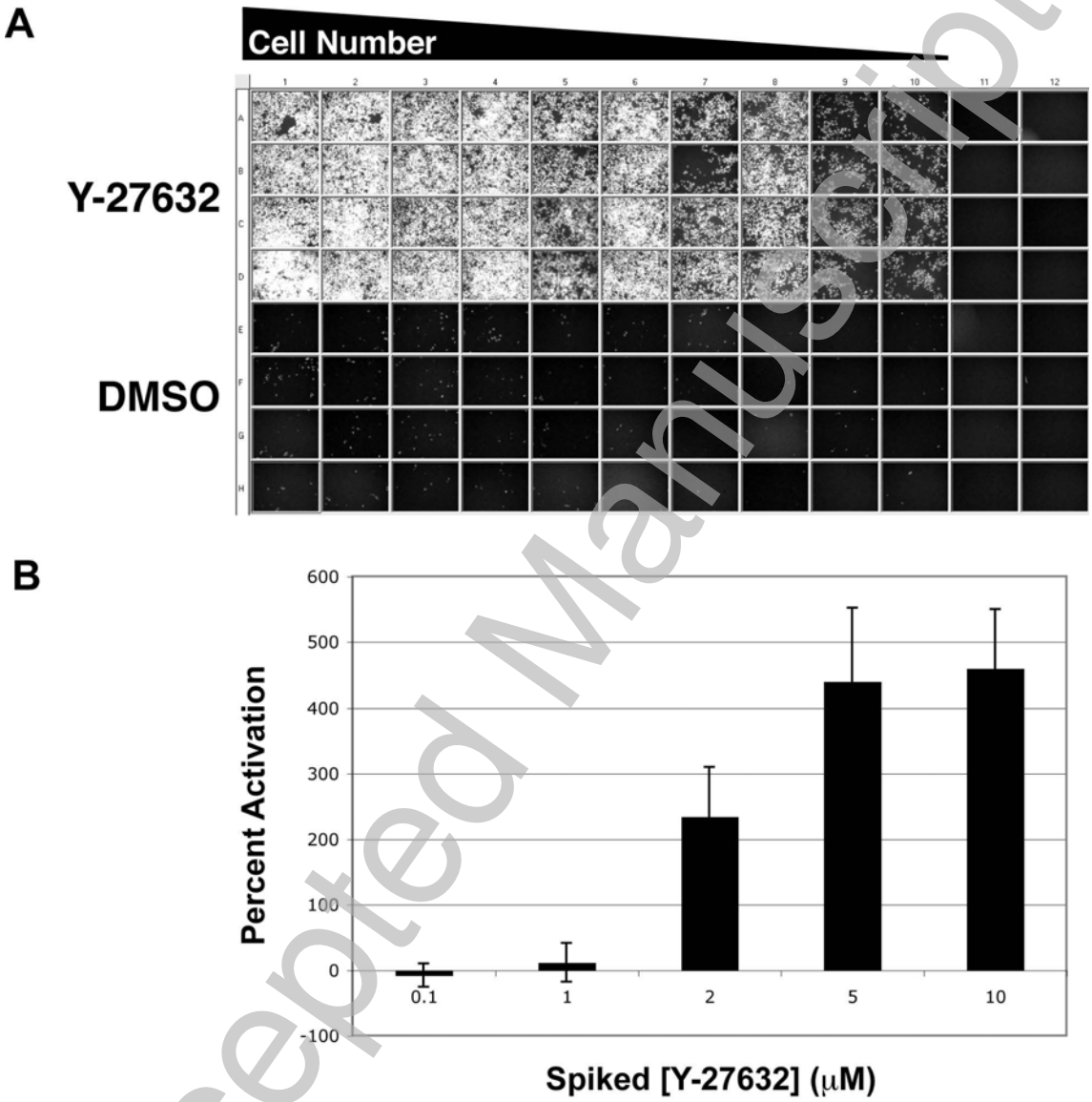

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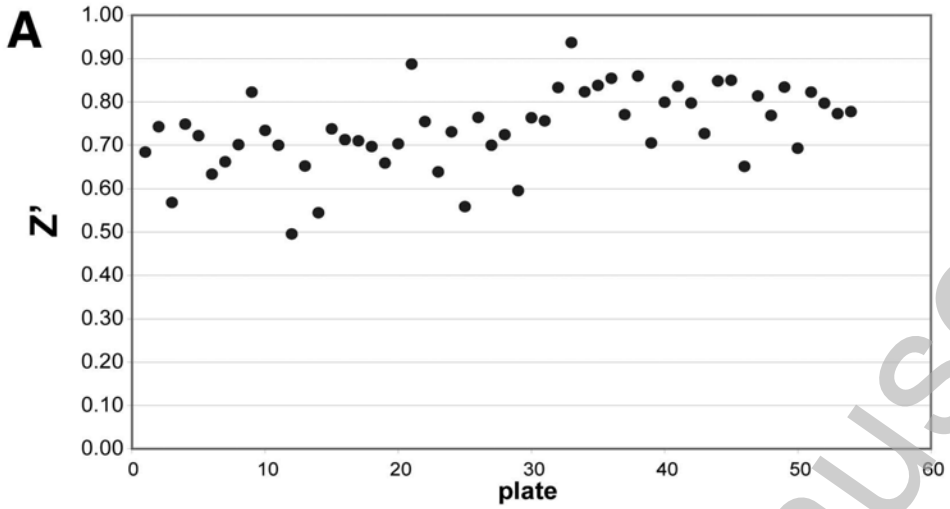
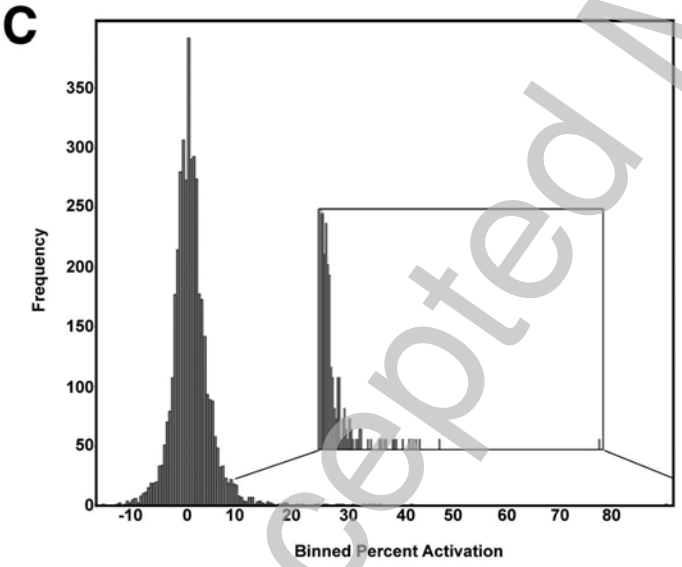
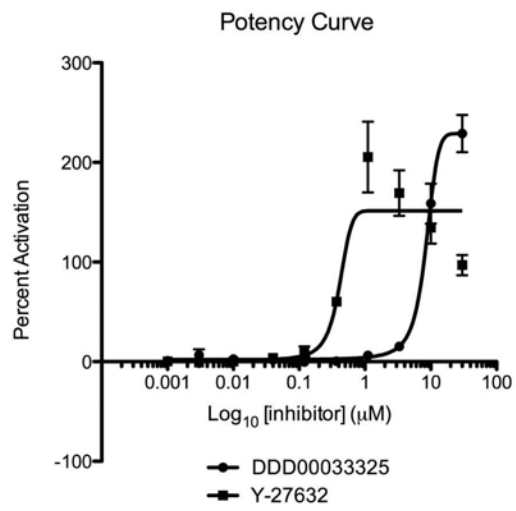


B

Performance Indicator	Average Value
Signal: Background	25.43 ± 19.5
% CV (high ctrl)	5.9 ± 2.3
Z'	0.76 ± 0.09
Mean IC_{50} Y-27632	0.98 ± 0.15





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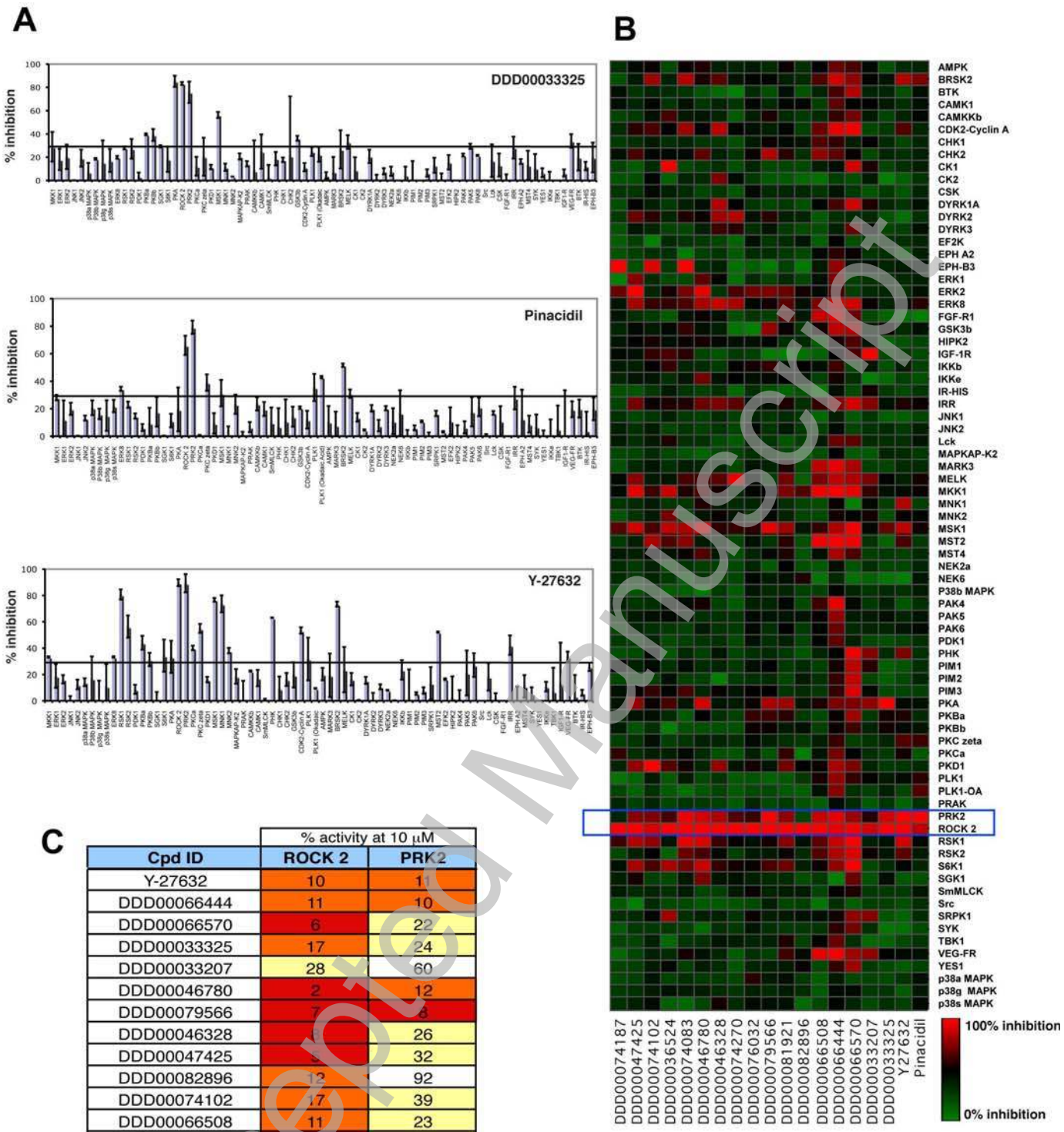

C

Cpd ID	% activity at 10 μM ROCK 2	PRK2
Y-27632	10	11
DDD00066444	11	10
DDD00066570	6	22
DDD00033325	17	24
DDD00033207	28	60
DDD00046780	2	12
DDD00079566	7	8
DDD00046328	1	26
DDD00047425	1	32
DDD00082896	13	92
DDD00074102	17	39
DDD00066508	11	23
DDD00081921	8	32
DDD00036524	6	45
DDD00074187	16	85
DDD00074083	8	11
DDD00076032	10	61
DDD00074270	15	49
pinacidil	34	21
DDD00066134	98	95

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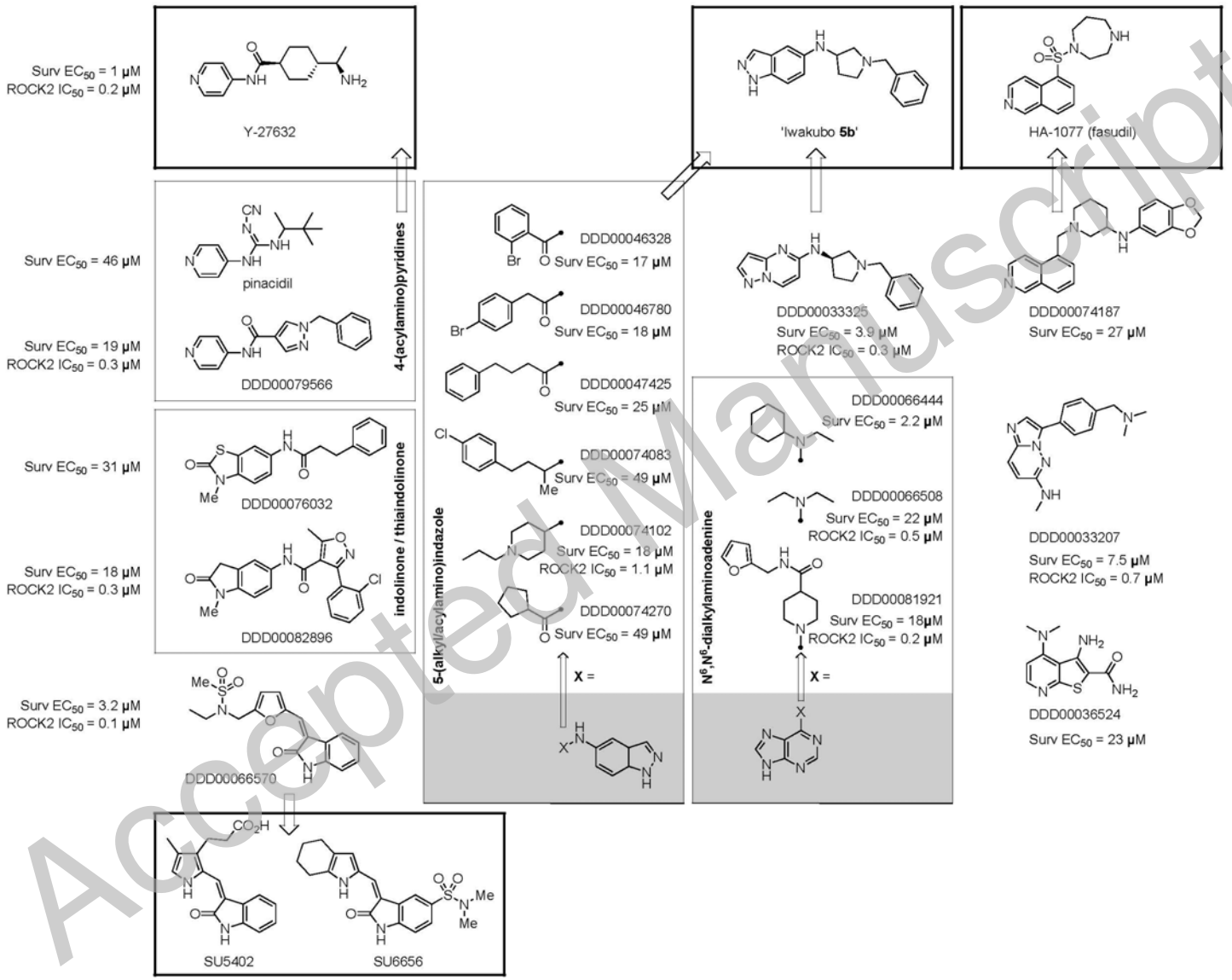

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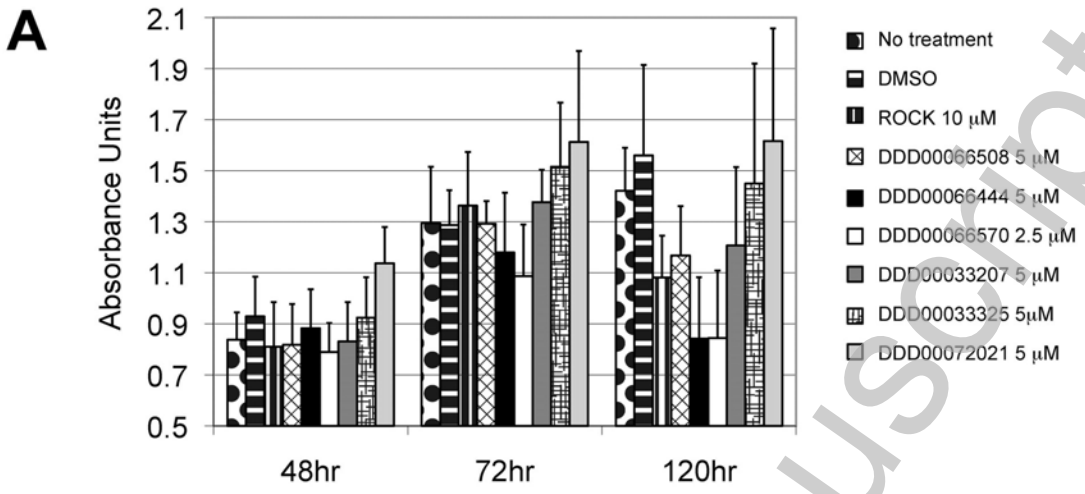

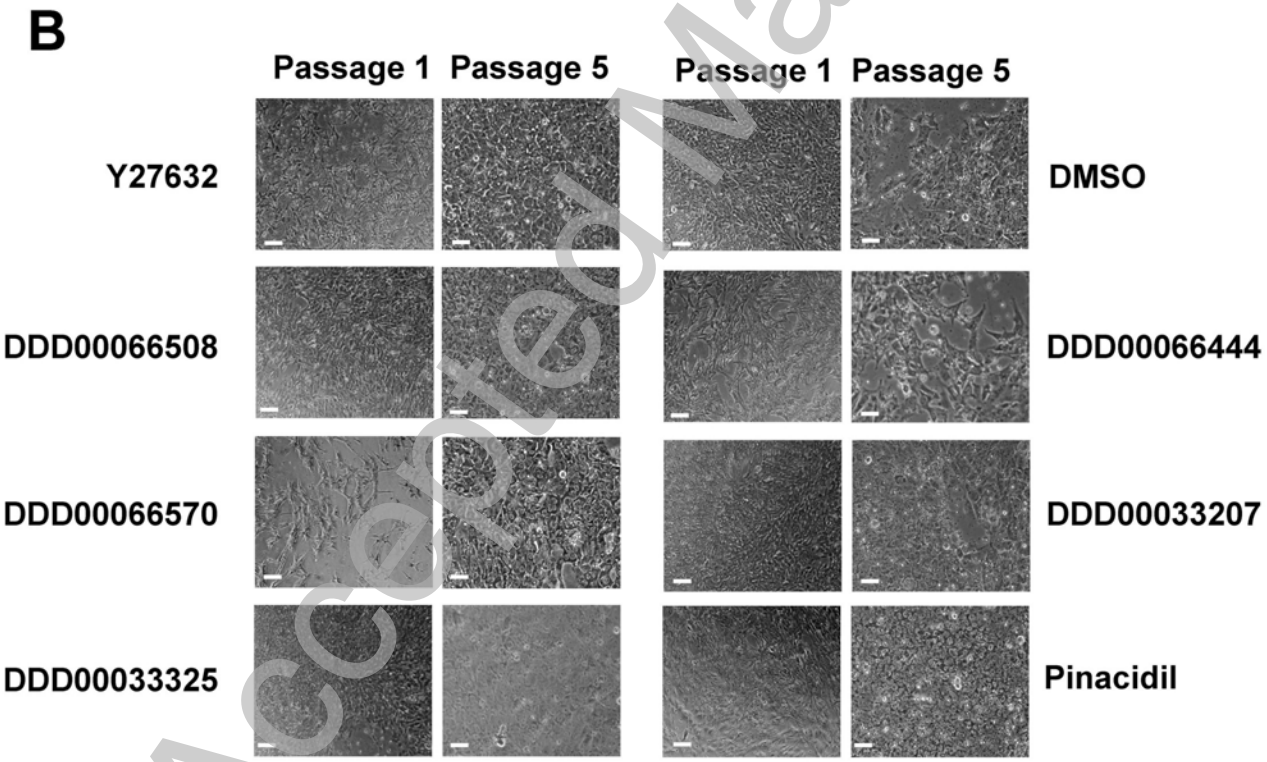

Figure 5 Andrews et al

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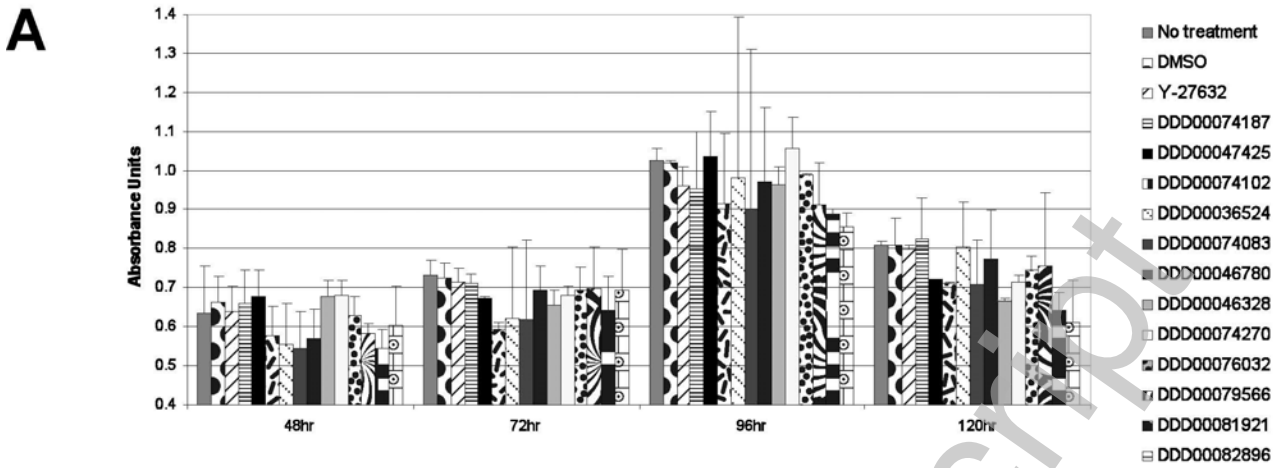

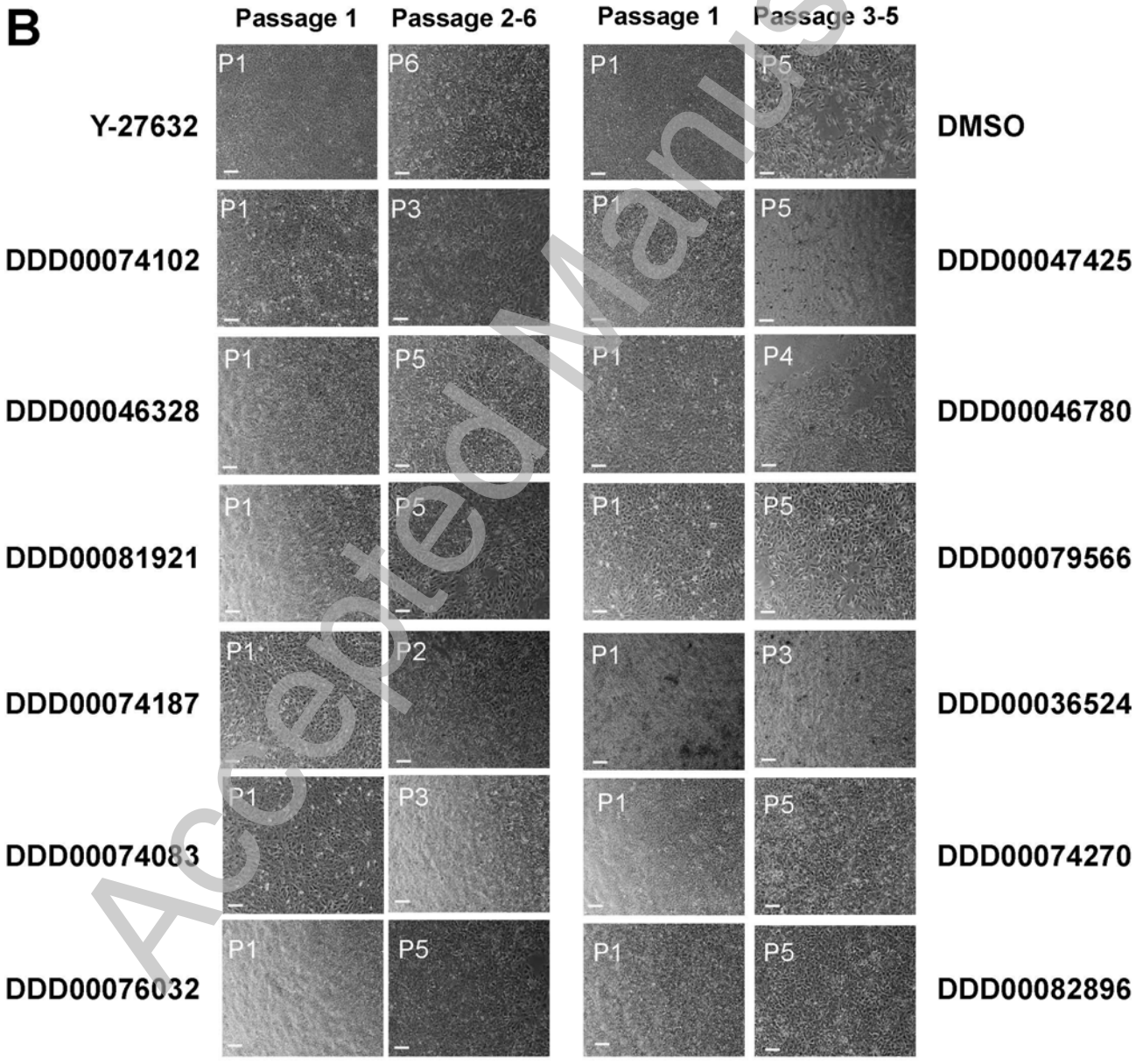

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A

	Pluripotency				Differentiation								
	Oct3/4	Sox2	Nanog	DNMT3B	hCG	Eomes	GATA4	GATA6	Sox17	Brachury	Pax6	Nestin	PECAM1
No treatment (P5)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
DMSO (P5)	0.76	0.92	0.88	0.79	0.76	0.79	0.67	0.81	0.53	0.46	0.52	0.53	0.11
ROCK (P5)	0.68	0.43	0.42	0.44	0.40	0.84	0.69	0.09	0.51	0.21	1.10	0.22	0.62
DDD00066508 (P5)	1.71	1.24	0.96	1.09	0.52	1.23	0.67	1.26	2.73	1.35	1.89	0.67	0.39
DDD00066444 (P5)	2.10	0.70	0.85	0.84	0.42	12.06	1.20	6.31	23.20	26.26	2.49	0.80	0.21
DDD00066570 (P1)	1.27	0.30	1.30	0.90	0.66	41.70	4.37	17.57	183.03	3.81	1.14	0.44	0.49
DDD00033207 (P5)	1.51	0.93	0.65	1.34	0.63	0.97	0.41	0.62	0.00	0.44	0.98	0.94	0.62
DDD00033325 (P5)	0.99	1.08	0.90	1.14	0.60	0.75	0.56	0.53	0.56	0.58	0.52	0.67	1.29
DDD00072021 (P5)	1.69	1.36	1.26	1.00	1.07	1.22	1.18	1.43	0.75	0.71	1.37	0.91	0.29

B

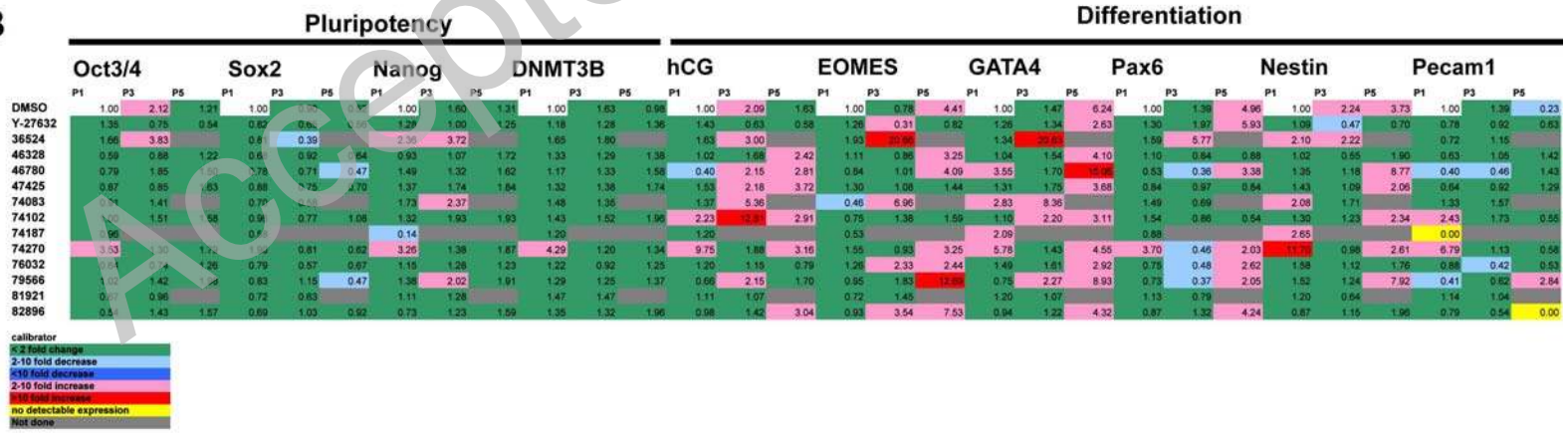

Figure 7 Andrews et al

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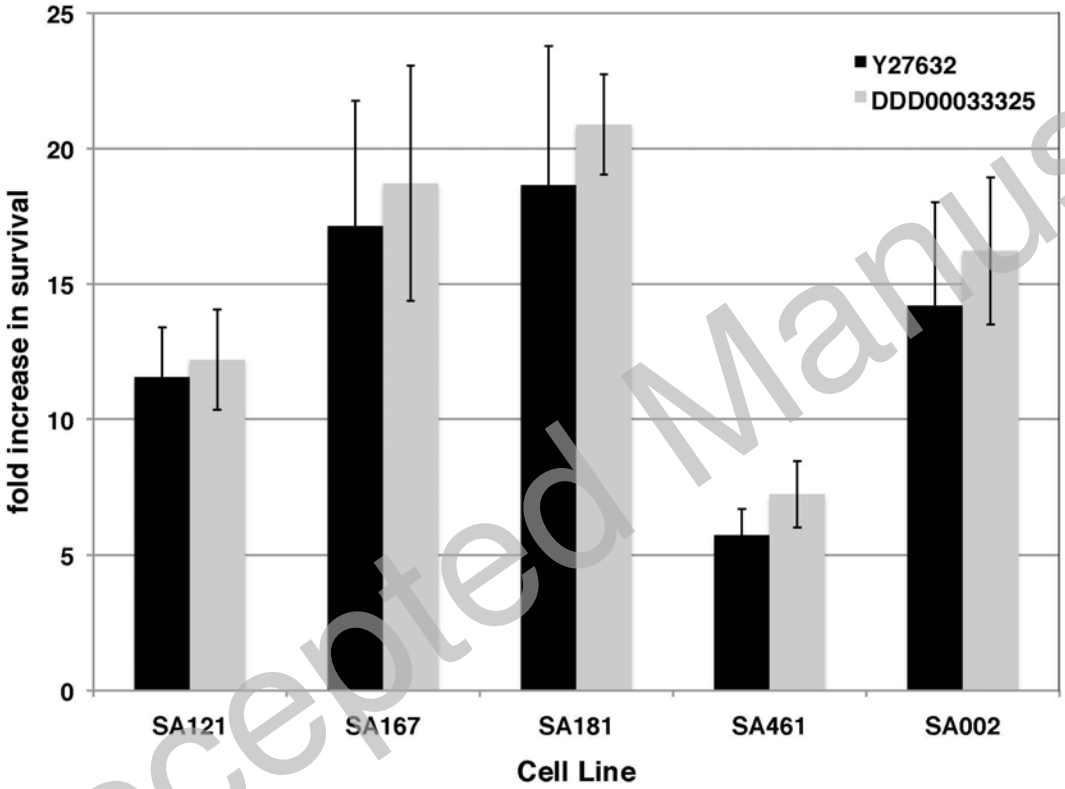

Fig. 8. Andrews et al.

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