Investigating the mechanisms of cell competition in mammals using *in vitro* systems

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Investigating the mechanisms of cell competition in mammals using *in vitro* systems

Cell competition leads to elimination of a viable cell population, by fitter cells. Despite over forty years of research, the molecular mechanisms of competition in mammals are poorly understood. During my PhD I have investigated the mechanisms of competition by exploring an established mammalian cell culture system, in which wild-type MDCK cells eliminate *scribble*-deficient cells, and I have also developed a novel cell culture system to model mammalian competition.

My work contributed to the discovery that *scribble*-deficient cells are eliminated not by biochemical exchange among cells, but by mechanical compaction. We termed this phenomenon mechanical competition. I employed transcriptional profiling to determine the molecular signature of mechanical losers, and identified activation of p53 signalling as their hallmark. My colleagues and I then demonstrated that elevation of p53 is both necessary and sufficient to trigger mechanical competition. In further investigating the mechanisms of mechanical competition, I found that compaction activates ROCK in *scribble*-deficient cells, and that this is required for their elimination. Inhibition of Src signalling in mechanical losers also protected them form out-competition, and integrin signalling is another pathway likely involved in mechanical competition.

While investigating p53 competition, we observed that p53-high and p53-low cells engage in directional migration, with p53-high cells always at the migrating front. As a side-project, I investigated the role of p53 in directional migration, by exploring an established model with a single leader cell and multiple followers. We established a method to generate multinucleated leaders on demand. By creating leaders from p53-deficient cells, I established that p53 signalling is required for some, but not all multinucleated cells to trigger collective migration, thus implicating p53 signalling in a type of migration involved in wound healing.

Finally, I successfully modelled p53-driven mechanical competition in a differentiated primary tracheal epithelial cell culture, thereby establishing a novel system to study mammalian competition, and also proving that p53 competition is conserved between different mammalian epithelia. Considering the involvement of p53, mechanical competition may play a major role in cancer.

Maja Goschorska

For my family

PREFACE

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University of similar institution except as declared in the Preface and specified in the text.

It does not exceed the prescribed word limit of 60 000 words, required by the Biological Sciences Degree Committee.

Maja Goschorska

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ABSTRACT

Cell competition leads to elimination of a viable cell population, by fitter cells. Despite over forty years of research, the molecular mechanisms of competition in mammals are poorly understood. During my PhD I have investigated the mechanisms of competition by exploring an established mammalian cell culture system, in which wild-type MDCK cells eliminate *scribble*-deficient cells, and I have also developed a novel cell culture system to model mammalian competition.

My work contributed to the discovery that *scribble*-deficient cells are eliminated not by biochemical exchange among cells, but by mechanical compaction. We termed this phenomenon mechanical competition. I employed transcriptional profiling to determine the molecular signature of mechanical losers, and identified activation of p53 signalling as their hallmark. My colleagues and I then demonstrated that elevation of p53 is both necessary and sufficient to trigger mechanical competition. In further investigating the mechanisms of mechanical competition, I found that compaction activates ROCK in *scribble*-deficient cells, and that this is required for their elimination. Inhibition of Src signalling in mechanical losers also protected them form out-competition, and integrin signalling is another pathway likely involved in mechanical competition.

While investigating p53 competition, we observed that p53-high and p53-low cells engage in directional migration, with p53-high cells always at the migrating front. As a side-project, I investigated the role of p53 in directional migration, by exploring an established model with a single leader cell and multiple followers. We established a method to generate multinucleated leaders on demand. By creating leaders from p53-deficient cells, I established that p53 signalling is required for some, but not all multinucleated cells to trigger collective migration, thus implicating p53 signalling in a type of migration involved in wound healing.

Finally, I successfully modelled p53-driven mechanical competition in a differentiated primary tracheal epithelial cell culture, thereby establishing a novel system to study mammalian competition, and also proving that p53 competition is conserved between different mammalian epithelia. Considering the involvement of p53, mechanical competition may play a major role in cancer.

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Chapter 1. INTRODUCTION

1.1. Cell competition

The term "cell competition" is used to describe a phenomenon where two viable cell populations compare their relative fitness levels and as a result the less fit cells are eliminated and replaced by the fitter cells (Fig.1.1). Cell fitness is broadly understood as the capacity to reproduce and populate a tissues, but the exact parameters that define cells as losers or winners are still not clear. Cell competition is a beneficial process that improves the performance of tissues by eliminating damaged, stressed, or malfunctioning cells, but it can also be hijacked by transformed cells and promote tumorigenesis.

1.1.1. The discovery of cell competition

The discovery of cell competition was serendipitous. Over 40 years ago Morata and Ripoll (1975) studied a set of mutations collectively called *Minute*. The *Minute* phenotype is caused by deficiencies in the production of ribosomal proteins. While homozygous mutations are lethal, heterozygous *Drosophila* melanogaster are delayed in development, but viable. When wild-type and *Minute*$^{+/-}$ clones were simultaneously generated in wild-type wing imaginal discs of *Drosophila* larvae, wild-type clones grew bigger than their *Minute*$^{+/-}$ counterparts. This might have been simply caused by a difference in proliferation rates between the two cell types. However, *Minute*$^{+/-}$ clones induced early in development were not recovered in adult flies, posing a question as to their fate (Morata & Ripoll 1975). The elimination of autonomously-viable mutant cells from mosaic tissues was termed cell competition (Fig.1.1). Further studies on mosaic imaginal discs

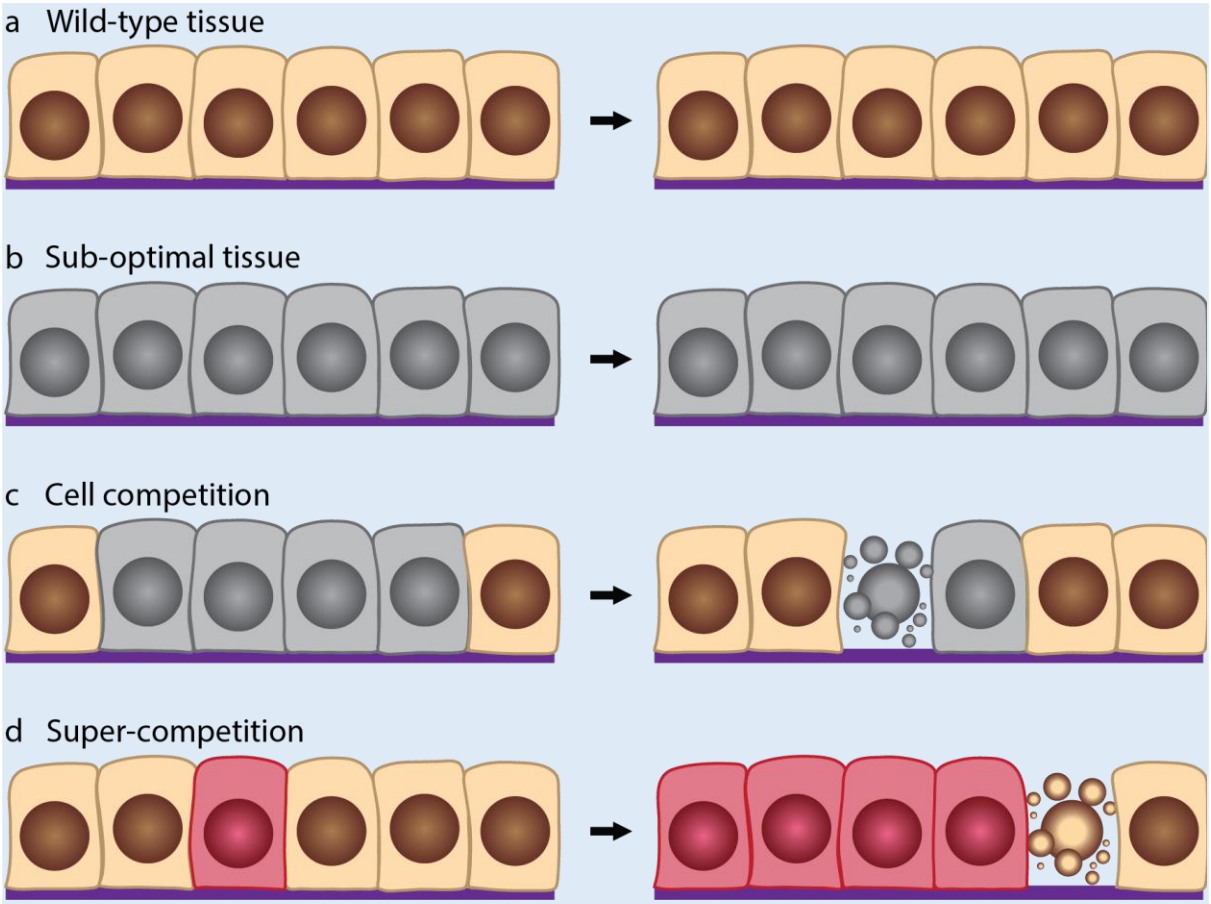


Figure 1.1. Cell competition.
Wild-type (brown, **a**) and sub-optimal (grey, **b**) cells are viable in homotypic tissues, but when juxtaposed in mosaic epithelia (**c**), the sub-optimal cells are killed and replaced by wild-type cells. (**d**) Super-competitors (red) kill and replace wild-type cells.

showed apoptosis of *Minute*$^{+/-}$ cells in clones surrounded by wild-type cells (Fig.1.1c). Inhibition of apoptosis rescued the mutant clones (Moreno *et al*. 2002).

Minute competition become the archetype of cell competition. These and subsequent studies established several features characteristic for this phenomenon: (1) Loser cells are viable in homotypic tissues. (2) Cell competition eliminates cells based on relative and not absolute levels of fitness. For instance, cells with a mild *Minute* phenotype are eliminated by wild-type cells, but can in turn eliminate cells with a stronger *Minute* phenotype (Simpson 1979; Simpson & Morata 1981). (3) *Minute* cell competition does not cross compartmental boundaries. Wild type cells from one developmental compartment will not be able to eliminate *Minute*$^{+/-}$ cells

placed immediately across a compartmental boundary (Morata & Ripoll 1975, reviewed by Amoyel & Bach 2014). (4) Cell competition does not alter the size or shape of a compartment (Simpson & Morata 1981). (5) Elimination of loser cells is accompanied by compensatory proliferation of winner cells.

Although *Minute* competition remains the golden standard of the phenomenon, in later years the definition of cell competition broadened, and not all of the above criteria are necessarily met by all of the established competition models. E.g. Crumbs competition crosses compartmental boundaries and affects tissue size and shape (Hafezi *et al*. 2012). For the purpose of this work I adapted a broad definition, where cell competition includes all phenomena leading to the elimination of a viable cell population by a fitter cell population (Fig.1.1).

1.1.2. Super-competition

Minute$^{+/-}$ flies are delayed in development and produce short, thin bristles. This phenotype is reproduced by loss of dMyc (Johnston *et al*. 1999), a *Drosophila* homologue of the mammalian transcription factor and proto-oncogene cMyc. dMyc is a critical growth regulator. Since, among numerous other effects, dMyc controls translation, it was proposed that, like *Minute*$^{+/}$, loss of dMyc should trigger cell competition in mosaic imaginal discs (Johnston *et al*. 1999). Indeed, although viable on their own, dMyc$^{-/-}$ clones grew very poorly in a dMyc$^{+/-}$ background when compared to simultaneously induced dMyc$^{+/+}$ clones, and were often completely eliminated (Johnston *et al*. 1999). Like *Minute*$^{+/-}$ cells, dMyc mutant cells surrounded by wild-type cells were eliminated by apoptosis (Moreno & Basler 2004).

Surprisingly, clones of cells with constitutively boosted dMyc signalling were able to eliminate wild-type cells (Moreno & Basler 2004; de la Cova *et al*. 2004). This was the first example of super-competition, a phenomenon in which wild-type cells behave as losers, and mutant cells as winners

(Fig.1.1d). Unlike the elimination of mutant losers, this type of competition can be potentially harmful and promote tumorigenesis.

1.2. **Mutations that trigger cell competition**

For decades, *Minute*$^{+/-}$ cells and dMyc-overexpressing cells remained respectively the archetypal losers and the model super-competitors. Because both ribosomal proteins and dMyc are involved in translation, and because of the many similarities between *Minute* and dMyc competition, they are often considered as two ways to model the same phenomenon. In concurrence, cells that overexpress dMyc lose their ability to out-compete wild-type cells when they are also *Minute*$^{+/-}$, suggesting a common pathway (Moreno & Basler 2004). Nevertheless, differences between *Minute* and Myc competition have also been reported. For instance *Minute*$^{+/-}$ cells were out-competed by wild-types in post-mitotic *Drosophila* follicular epithelia, but neither deficiency nor ever-expression of dMyc induced competition (Tamori & Deng 2013).

Other models of cell competition have been gradually introduced over the years, but it was only the last decade that saw an explosion of the field and the identification of numerous novel mutations able to trigger competition (Table.1.1). These recent studies as yet failed to identify any molecule or signalling pathway involved in all types of competition, suggesting that there may be more than one independent mechanism for eliminating less fit cells, and thus more than one type of cell competition. It is not yet known how many types of competition exist, and which mutations eliminate loser cells by common pathways.

Table 1.1. Triggers of cell competition.
Presents examples of genes and pathways that, when misregulated, turn cells into losers or into super-competitors.

Factor	Role in competition	Experimental model	References
Minute	*Minute*$^{+/-}$ results in loser status	*Drosophila* imaginal discs, *Drosophila* adult gut, *Drosophila* follicle cells, Mouse embryo	Morata and Ripoll, 1975; Simpson, 1979; Simpson and Morata, 1981; Moreno *et al.*, 2002; Oliver *et al.*, 2004; Tyler *et al.*, 2007; Tamori and Deng, 2013; Kolahgar *et al.*, 2015
Myc	Deficiency results in loser status; Over-activation generates super-competitors	*Drosophila* imaginal discs, Mouse embryo, Mouse ES cells	Johnston *et al.*, 1999; de la Cova *et al.*, 2004; Moreno and Basler, 2004; Senoo-Matsuda and Johnston, 2007; Claveria *et al.*, 2013; Sancho *et al.*, 2013)
BMPs	Deficiency results in loser status	*Drosophila* imaginal discs, Mouse embryo, Mouse ES cells	Moreno *et al.*, 2002; Sancho *et al.*, 2013)
Ras	Deficiency results in loser status in *Drosophila*; Over-activation generates super-competitors in *Drosophila* and losers in MDCK cells	*Drosophila* imaginal discs, MDCK cells	Karim and Rubin, 1998; Prober and Edgar, 2000; Hogan *et al.*, 2009
Src	Over-activation results in loser status	MDCK cells, zebrafish embryos	Vidal *et al.*, 2006; Vidal *et al.*, 2010; Kajita *et al.*, 2010; Kajita *et al.*, 2014
Scribble	Deficiency results in loser status	*Drosophila* imaginal discs, MDCK cells	Brumby and Richardson, 2003; Igaki *et al.*, 2006; Chen *et al.*, 2012; Norman *et al.*, 2012, Wagstaff *et al.*, 2016
Disc large	Deficiency results in loser status	*Drosophila* imaginal discs	Igaki *et al.*, 2006; Ohsawa *et al.*, 2011
Lethal giant larvae	Deficiency results in loser status	*Drosophila* imaginal discs	Igaki *et al.*, 2006; Grzeschik *et al.*, 2007; Menendez *et al.*, 2010; Tamori *et al.*, 2010
Mahjong	Deficiency results in loser status	*Drosophila* imaginal discs, MDCK cells	Tamori *et al.*, 2010; Tamori and Deng, 2013
Crumbs	Over-expression generates super-competitors; Deficiency results in loser status	*Drosophila* imaginal discs	Hafezi *et al.*, 2012
Yorkie/ Yap	Over-expression generates super-competitors in *Drosophila* and NIH3T3 cells and losers in MDCK cells; Deficiency results in loser status in *Drosophila* and NIH-3T3 cells	*Drosophila* imaginal discs	Tyler *et al.*, 2007 Ziosi *et al.*, 2010 Mamada *et al.*, 2015 Chiba *et al.*, 2016
Wingless pathway	Inhibition results in loser status; Over-activation generates super-competitors	*Drosophila* imaginal discs, *Drosophila* adult gut	Vincent *et al.*, 2011; Suijkerbuijk *et al.*, 2016
JAK/STAT pathway	Inhibition results in loser status; Over-activation generates super-competitors	*Drosophila* imaginal discs	Rodrigues *et al.*, 2012

1.2.1. Myc-dependent competition models

Whenever a new competition model is established, it is customary to test whether it is related to Myc and/or *Minute* competition. An example of a Myc-dependent competition is the elimination of clones deficient for the GTPase Ras1 from mosaic *Drosophila* wing imaginal discs. Loss of Ras1 led to the elimination of the mutant clones by apoptosis. Conversely, activation of Ras1 increased the size of mutant clones (Karim & Rubin 1998; Prober & Edgar 2000). Like dMyc, loss of Ras1 resulted in smaller, slower growing cells, while overexpression of either protein accelerated cell growth and proliferation rates. Prober and Edgar (2000) decided to test whether these similarities are a result of Ras1 controlling cells growth and proliferation via dMyc. Indeed, over-activation of Ras1 elevated dMyc in wing imaginal discs. Moreover, clones over-expressing dMyc behaved similarly to Ras1-deficient clones that also overexpressed dMyc, suggesting both that dMyc signals downstream of Ras1 and that dMyc is required for Ras1 competition (Prober & Edgar 2000).

Another examples of Myc-dependent competition stems from studies on the bone morphogenetic protein (BMP). In mosaic *Drosophila* wing imaginal discs (Burke & Basler 1996) and in mouse embryos (Sancho *et al*. 2013), cells with a deficiency in BMP signalling are eliminated by apoptosis by wild-type cells, and this depends on Myc. A Myc-linked model of competition that gained the most attention in recent years is Hippo pathway competition (Tyler *et al*. 2007; Ziosi *et al*. 2010; Neto-Silva *et al*. 2010; Mamada *et al*. 2015; Chiba *et al*. 2016).

1.2.2. Hippo pathway and cell competition

The Hippo-Salvador-Warts pathway is a major and universal regulator of organ size and a tumour suppressor pathway (Lu *et al*. 2010). It is activated by high cell density and by cell contact, and inhibits the transcriptional co-factor Yorkie in *Drosophila*, or YAP and TAZ in mammals. Inhibition of Yorkie

in turn prevents transcription of a number proliferative and anti-apoptotic genes, including Myc (de Beco *et al*. 2012). The Hippo pathway was first linked to cell competition when a genetic screen in *Drosophila* imaginal discs revealed that inactivation of several of its components prevented out-competition of *Minute*$^{+/-}$ cells (Tyler *et al*. 2007). It has been further shown that inhibition of the Hippo pathway turned cells into super-competitors, increasing death of the surrounding wild-type cells (Tyler *et al*. 2007; Ziosi *et al*. 2010). Similarly, clones overexpressing Yorkie were noticeably larger than their wild-type twins, which gradually disappeared by apoptosis (Ziosi *et al*. 2010).

In *Drosophila* wing imaginal discs, Hippo pathway competition has been linked to Myc competition. dMyc was autonomously up-regulated in cells overexpressing Yorkie (Ziosi *et al*. 2010). When this difference in Myc levels was reduced, the ability of clones overexpressing Yorkie to out-compete wild-type cells was diminished (Ziosi *et al*. 2010). Yet, Hippo competition did not depend solely on Myc. Clones of cells that lost Yorkie were eliminated from wild-type tissues and overexpression of dMyc was not sufficient to rescue the mutant clones (Ziosi *et al*. 2010).

Yorkie competition is conserved in mouse NIH3T3 embryonic fibroblast cell culture (Mamada *et al*. 2015). Cells with reduced activity of the Yap-target Taed became losers and died by apoptosis, whereas cells with increased Tead activity became super-competitors. Moreover, Tead directly controlled expression of Myc and overexpression of Myc was sufficient to generate winner cells (Mamada *et al*. 2015).

In a surprising reversal to the above reports, Chiba *et al*. (2016) recently reported that mammalian epithelial MDCK cells over-expressing Yap acted as losers, rather than winners, and were eliminated from wild-type monolayers by apical extrusion. The authors did not test the involvement of Myc in this competition model. Notably, when Yap-overexpressing cells were juxtaposed with cells overexpressing K-Ras (G12V) or v-Src they switched from losers to winners (Chiba *et al*. 2016). This suggests that the

role of Yap in cell competition depends on the status of neighbouring cells and potentially explains the discrepancies between this and previous studies.

1.2.3. Polarity factors and cell competition

A variety of polarity factors have been linked to cell competition. For instance in *Drosophila*, epithelial cells that lose the regulators of apico-basal polarity Letahl giant Larvae (*lgl*), Disc large (*dlg*) or *scribble* are eliminated by normal cells. In contrast to *Minute* and Myc competition mutant *Drosophila* are not viable. Moreover, in mosaic tissues the mutant clones are only eliminated after showing profound morphological defects. These discrepancies from the archetypal modes of competition posed the question whether the elimination of cells with polarity defects should be referred to as cell competition (Levayer & Moreno 2013). Since mutant imaginal disc cells are viable (if abnormal) on their own but are eliminated from mosaic tissues, meeting the broadest definition of cell competition, for the purpose of this work I chose to classify this phenomenon as cell competition.

In *Drosophila*, *lgl*, *dlg* and *scribble* act as neoplastic tumour suppressors, as their mutants lose polarity forming multi-layered amorphous growths. In wing imaginal discs mutant clones surrounded by normal cells were eliminated by apoptosis (Brumby & Richardson 2003; Grzeschik *et al*. 2007; Menéndez *et al*. 2010; Tamori *et al*. 2010; Ohsawa *et al*. 2011; Chen *et al*. 2012), providing an example of a tumour-suppressive role of cell competition. *scribble*-induced competition in mammalian cells is a particular focus of my studies and will be described in detail in a later chapter.

1.2.4. Crumbs and cell competition

Crumbs is an apical determinant in apico-basal polarity and another example of a polarity factor involved in cell competition. However, unlike *lgl*, *dlg* and *scribble*, deficiency in Crumbs generates winners and not losers.

Its role in competition was discovered when Hafezi and colleagues (2012) carried a genetic screen in mosaic *Drosophila* eyes, looking for competitive winners. In homotypic tissues, cells overexpressing crumbs became more rounded and formed neoplastic overgrowths, but were viable. In mosaic tissues lowering the levels of crumbs created super-competitors, while clones overexpressing crumbs acted as losers and were eliminated by apoptosis.

Like other polarity factor competition models, Crumbs competition does not follow all rules established for *Minute* and Myc competition. For instance, crumbs competition can cause apoptosis across compartmental boundaries. It also affects tissue size and shape. (Hafezi *et al*. 2012). This suggests that crumbs competition may be an alternative type of competition, independent of *Minute* or Myc competition. Indeed, Hafezi *et al*. (2012) did not observe any differences in the levels of Myc between mutant and wild-type clones. Similarly, levels of crumbs did not vary between wild-type and Myc-overexpressing cells.

1.2.5. Ras and Src in cell competition

Ectopic activation of Ras or Src in mammalian MDCK cell culture provided a proliferative advantage, yet the mutant cells were extruded from the epithelial monolayer when co-cultured with wild-type cells (Hogan *et al*. 2009; Kajita *et al*. 2010). These results contrast with the super-competition status of cells over-expressing Ras1 in *Drosophila* (Karim & Rubin 1998; Prober & Edgar 2000), but are similar to observations on dSrc-transformed cells (Vidal *et al*. 2006; Vidal *et al*. 2010). In mammalian epithelia, Myosin-II activity is upregulated selectively in the transformed cells surrounded by normal cells. This in turn induces activation of Rho/ Rho kinase signalling and accumulation of filamin A and vimentin in the normal cells at the interface with mutant cells, generating a contractile force that leads to apical extrusion of the transformed cells. Knockdown of filamin A or vimentin in normal MDCK cells, and filamin-deficiency in zebrafish embryos,

profoundly suppressed apical extrusion of the neighbouring transformed cells (Kajita *et al*. 2014).

1.2.6. *Wnt* signalling and cell competition

The *Wnt* pathway is involved in tissue patterning, cell proliferation, cell fate specification and survival, and is often disrupted in cancer, particularly in intestinal and liver tumours. The *Drosophila Wnt* homologue wingless is involved in patterning the wing imaginal discs, where it promotes expansion of the wing pouch during disc development (de Beco *et al*. 2012). Work from my lab and the Vincent group has shown that the function of wingless as a survival pathway in wing discs may not be cell-autonomous (Vincent *et al*. 2011). When wingless or its receptor are removed from whole compartments, the cells survive. In contrast, mutant cells are eliminated by apoptosis by wild-type in mutant tissue, meeting the criteria of cell competition. While loss of wingless signalling turn cells into losers, over-activation of the pathway confers a competitive advantage. Mutations in APC and axin, both of which are negative regulators of wingless, create super-competitors.

Wnt competition depends on notum, a secretory glypican phospholipase that controls wingless signalling in a negative feedback loop. In *Drosophila* wing imaginal discs the loss of axin over-activates wingless signalling, which induces secretion of notum. In mosaic tissues the secreted notum will then inhibit wingless signalling in the neighbouring wild-type cells, but cannot affect the constitutively-active wingless signalling of the mutant cells. Thereby, notum exacerbates the difference in Wingless signalling between the two cell populations. Loss of notum prevents axin mutant cells from acting as super-competitors (Vincent *et al*. 2011).

Wnt competition seems to function independently from *Minute* or Myc competition. Not only are Myc levels not elevated in super-competitors with over-activated wingless signalling, but high levels of wingless signalling

actually supress the expression of Myc. This way, in a striking reversal to Myc competition, in *Wnt* competition the loser cells have higher levels of Myc than the winners (Vincent *et al*. 2011). To further test the involvement of Myc in *Wnt* competition, Vincent and colleagues (2011) equalised the levels of Myc by either inhibiting or overexpressing dMyc throughout the tissue. Neither affected *Wnt* competition, confirming that it does not depend on Myc competition. Increasing Myc expression in the host tissue also did not rescue it from out-competition by APC-mutant adenomas in adult *Drosophila* intestine (Suijkerbuijk *et al*. 2016). *Wnt* competition is also unrelated to *Minute* competition, as the two types of competitive interactions are additive, and as the loss of notum does not prevent *Minute* competition (Vincent *et al*. 2011).

1.2.7. JAK/STAT pathway and cell competition

The JAK/STAT cytokine pathway, among its other functions, is a crucial regulator of growth. Over-activation of Stat9e2E, the only transcription factor regulated by the pathway in *Drosophila*, causes a dramatic overgrowth of the eye. In mosaic wing and eye imaginal discs, as expected from cell competition, the viability of cells lacking Stat92E is context-dependent. While the clones can survive in a *Minute*$^{+/-}$ background, they are out-competed by wild-type cells and eliminated by apoptosis. Conversely, cells with sustained activation of Stat92E become super-competitors and trigger apoptosis of wild-type cells within several cell diameters form the mutant clones (Rodrigues *et al*. 2012). Like many other types of competition, JAK/STAT competition does not cross compartment boundaries.

Rodrigues and colleagues (2012) proposed that JAK/STAT competition is an independent type of cell competition, parallel to other known models. They reported no elevation in Myc, no activation of Yorkie, Wingless or Decapentaplegic signalling, and no increase in ribosome biogenesis in cells with hyper-activated Stat92E signalling, suggesting that none of those

pathways are required for those cells to become super-competitors. Similarly, they did not observe hyper-activation of Stat92E in cells with elevated dMyc levels or over-activated Wingless signalling, indicating that Stat92E is not a downstream effector in these types of competition. In contrast, a recent study reported that JAK/STAT signalling is involved in *Minute* competition in adult *Drosophila* gut, where it is up-regulated in competing wild-type cells (Kolahgar *et al*. 2015). This increase in JAK/STAT signalling promotes proliferation and propagation of the wild-type cells during competition.

1.2.8. p53 in cell competition

The role of p53 signalling in the elimination of loser cells appears complex and dependent on the competition model. The involvement of p53 in the elimination of mammalian epithelial cells was a major topic of my studies.

The primary function of p53 is to coordinate transcriptional responses to stress. By triggering cell cycle arrest, senescence or apoptosis, p53 facilitates repair or elimination of damaged, and potentially dangerous cells (Muller & Vousden 2014). The key protective function of p53 is emphasized by the fact that approximately half of all human tumours carry mutant p53. In absence of stress, p53 is held at low levels, primarily by binding to the E3 ubiquitin ligase Mdm-2. Mdm-2 sequesters p53 it in the cytosol and targets it for proteasomal degradation (Muller & Vousden 2014). In response to a wide range of intracellular and extracellular stressors, p53 is stabilised by post-translational modifications, and released from its complex with Mdm-2. It can then translocate to the nucleus, where it acts as a transcription factor. Alternatively, the stabilised p53 can also perform extra-nuclear functions, such as activating apoptosis by directly regulating mitochondrial proteins (reviewed by Amaral *et al*. 2010).

p53 plays a critical role in cell-autonomous removal of malfunctioning cells. This response is triggered based on the absolute level of cell stress or

damage. For instance DNA damage that exceeds a certain threshold will commit cells to a p53-dependent apoptosis or cell cycle arrest. It has been hypothesized that cell competition provides a mechanism for the elimination of cells that are stressed, but not sufficiently so to trigger a p53 response in homotypic tissues (Bondar & Medzhitov 2010). This way viable but malfunctioning cells could be eliminated based on relative rather than absolute p53 levels, improving tissue fitness.

To verify this hypothesis Bondar and Medzhitov (2010) studied the interaction of cells with different levels of p53 activity in mouse bone marrow. First, they mildly boosted p53 levels in one cell population either by subjecting donor animals to sub-lethal levels of ionising irradiation, or by genetically reducing the levels of the p53-inhibitor Mdm2. They then introduced a mix of stressed and untreated cells into lethally irradiated host animals. In time, without altering the total number of cells, the contribution of cells from untreated animals increased at the expense of damaged cells, suggesting an ongoing cell competition.

To more directly test the effect of p53 status on cell fate in mosaic bone marrow, Bondar and Medzhitov (2010) genetically introduced clones of cells expressing a dominant negative form of p53 in otherwise normal tissues. Basal levels of stress *in vivo* were too low to activate p53 signalling and trigger competition between the two cell populations. However, when mild irradiation induced p53 in normal cells, the irresponsive mutant cells gained a competitive advantage. Together, all of the above data suggests that different levels of stress (in this case irradiation) experienced by cells translate to different levels of activation of p53, and that those differing levels of p53 turn the more stressed cells into competitive losers. Unlike other known competition models, p53 competition requires and additional, external trigger (irradiation), suggesting that p53 may play a more downstream, effector role, than the other characterised mutations. Very similar *in vivo* studies were also conducted by Marusyk and colleagues, who

reported that ionizing irradiation selects for p53-deficient hematopoietic progenitors (Marusyk *et al*. 2010).

Microarray analysis on a number of regulators of cell proliferation indicated a boost in growth of p53-deficient cells in mosaic tissues, consistent with compensatory proliferation of winner cells, characteristic for many competition models (Bondar & Medzhitov 2010). The same study revealed that $p16^{INK4a}$ and other markers of senescence were up-regulated in competing loser cells, suggesting competition-induced senescence. Indeed, expansion of p53-deficient cells was reduced if senescence was blocked in the loser cells (Bondar & Medzhitov 2010). Surprisingly, blocking death did not affect competition, suggesting that, unlike in many competition models, it is senescence that leads to the elimination of loser cells from stressed bone marrow.

While Bondar and Medzhitov (2010) demonstrated the importance in p53 signalling in the loser cells, others looked at the function of p53 in winner cells. When de la Cova and colleagues (2014) probed for the molecular basis of the fitness of Myc super-competitors in *Drosophila*, they observed that overexpression of Myc induced p53. Therefore, in this competition model, it is the winner and not the loser cell population that shows elevated p53 signalling. They further reported that while in monoculture p53 somewhat countered the effects of Myc by suppressing glycolysis, during competition it further boosted the glycolytic flux in the mutant cells, thereby enhancing their fitness and promoting expansion. Whereas the loss of p53 in the mutant cells had no significant effect in absence of competition, it impaired metabolism and triggered apoptosis in competing Myc-overexpressing cells and prevented them from eliminating the wild-type cells (de la Cova *et al*. 2014). In contrast, p53-null cells eliminated $Minute^{+/-}$ cells as effectively as wild-type cells, indicating that p53 signalling in the winner cell population is not a universal mechanism. What is more, loss of p53 in the $Minute^{+/-}$ cells did not protect them form out-competition by wild-type cells (Kale *et al*. 2015).

Our group has recently published a paper on p53 cell competition in mammalian cell culture (Wagstaff *et al*. 2016). The findings of this paper are the subject of several chapters of my Thesis.

1.3. **Mechanisms of cell competition**

Cell competition can be roughly divided into three phases: (1) A triggering event that alters the fitness of a cell population. The trigger can be a mutation, e.g. in one of the many genes described in the previous chapter. Alternatively, the change in fitness could have a non-genetic cause, such aging of a cell population (Oertel *et al*. 2006; Martins *et al*. 2014). (2) The recognition phase, in which two cell populations compare their relative fitness levels and communicate to establish their relative loser and winner status. (3) The effector phase, in which the winner cells propagate and the loser cells are eliminated.

1.3.1. Triggers of cell competition

As the number of mutations known to trigger cell competition increased, attempts have been made to identify common features of loser cells and of winner cells. A number of theories have been proposed, some of which may be complementary.

1.3.1.1. Proliferation rate as a trigger of cell competition

Decreased growth rate is a major characteristic of both *Minute* and Myc-deficient cells. Moreover, Simpson & Morata (1981) demonstrated that slower growing *Minute*$^{+/-}$ mutants are eliminated earlier. Therefore, it has been proposed that cell competition results from a difference in growth rates between two cell populations (Simpson & Morata 1981; Johnston *et al*. 1999; Moreno & Basler 2004). It has been argued that the reason why *Minute* competition is suppressed in starving animals (Simpson 1979), is because nutrient-deprived wild-type cells grow slower, losing their

advantage over *Minute*$^{+/-}$ cells. Similarly, the fact that competition stops in imaginal discs when developmental growth ceases (Simpson & Morata 1981) has been used to argue the importance of proliferation for cell competition.

A strong argument against proliferation as a standalone trigger of cell competition is that only some growth regulators can elicit this process. For example, although Dp110 and CycD + Cdk4 produce larger clones than normal cells, they do not affect the size of the surrounding clones, instead increasing the total size of a tissue (de la Cova *et al*. 2004; Hafezi *et al*. 2012). Similarly, loss of Tsc1, an inhibitor of growth through the target of rapamycin pathway, promoted growth but did not turn cells into super-competitors (Hafezi *et al*. 2012). Moreover, *Minute* and Mahjong competition was detected in post-mitotic *Drosophila* ovary follicular epithelia (Tamori & Deng 2013), and post-mitotic wild-type clones were able to kill surrounding *Minute*$^{+/-}$ cells in *Drosophila* midgut (Kolahgar *et al*. 2015), demonstrating that proliferation is not and absolute requirement for competition. Nevertheless, since differing proliferation rates are a common feature of many competition models, it is plausible that they are necessary but not stand-alone triggers of those types of competition.

1.3.1.2. The ligand capture model

One of the earliest hypothesis explaining the mechanism of cell competition was the ligand capture model, where cells compete for a limited supply of an extracellular survival factor. The winner cell population is more efficient at capturing the survival signal, e.g. by having more receptors or due to a higher endocytic rate. In mosaic tissues, the winner cells locally deplete the survival factor, thereby causing death of the under-supplied loser cells. This model has been suggested as an explanation for *Minute* and dMyc competition in the *Drosophila* wing imaginal disc (Moreno *et al*., 2002). The cells reportedly competed for Decapentaplegic (Dpp), a *Drosophila* homologue of BMP. Consistent with this theory, reduced Dpp signalling was

reported in competing *Minute*$^{+/-}$ and dMyc-deficient cells (Moreno *et al*. 2002; Moreno & Basler 2004; Tyler *et al*. 2007), and boosting Dpp signalling rescued Myc-deficient loser cells (Moreno & Basler 2004; Ziv *et al*. 2009). Nevertheless, the role of Dpp in cell competition remains highly controversial. De la Cova (de la Cova *et al*. 2004) and Martin (Martin *et al*. 2009) reported no reduction in Dpp signal in *Minute*$^{+/-}$ and dMyc-deficient loser cells. The strongest evidence against the Dpp ligand capture model comes from a recent *in vitro* study on mouse stem cells (Sancho *et al*. 2013). In a transwell system where cells with reduced BMP (mouse Dpp) signalling shared medium with normal cells, the mutant cells acted as losers. If the normal cells were out-competing the mutant cells by depleting the medium of BMP, supplementing the medium with additional BMP should have rescued the loser cells. This was not the case, indicating that while BMP is involved in cell competition, it does not do so via a ligand capture mechanism. Moreover, Sancho and colleagues reported that BMP competition requires cMyc signalling, thereby placing BMP up-stream of cMyc competition, rather than as its effector (Sancho *et al*. 2013).

1.3.1.3. Survival pathways and cell competition

Cell competition is triggered by mutations in many key developmental signalling pathways (reviewed by Amoyel & Bach 2014). Extracellular ligands of those pathways act as survival factors, i.e. signals that are essential for cells to live, and without which they would undergo apoptosis. Traditionally, it has been thought that the requirement for survival signalling is cell autonomous. However, studies on the role of survival pathways in competition challenge this assumption. For instance, as described in a previous section, cells with reduced wingless survival signalling are only eliminated when juxtaposed with more fit cells (Vincent *et al*. 2011). Similar observations were made for the JAK/STAT pathway (Rodrigues *et al*. 2012), for Ras signalling (Karim & Rubin 1998; Prober &

Edgar 2000) and for Decapentaplegic (BMP) signalling (Burke & Basler 1996; Sancho *et al*. 2013).

It is not known if reduction in every survival signal can turn cells into losers, and if all survival pathways trigger competition by a common downstream mechanism. It is however known that creating super-competitors is not as simple as over-activating a survival pathway. While cells that lost Insulin/PI3K pathway signalling are eliminated by competition (Böhni *et al*. 1999; Verdu *et al*. 1999), ectopic activation of PI3K does not trigger super-competition (de la Cova *et al*. 2004).

1.3.1.4. Stress as a trigger of cell competition

Cellular stress level is a compelling candidate for the mysterious determinant of cell fitness. There are several lines of evidence suggesting that differences in stress levels experienced by cells trigger cell competition. In particular, studies on cell competition in mouse bone marrow directly demonstrated that increasing stress levels by irradiation was sufficient to create a loser cell population, out-competed by normal cells (Bondar & Medzhitov 2010). As described in a previous section this type of competition was mediated by the central stress sensor p53 (Bondar & Medzhitov 2010). Another example of the involvement of stress-associated signalling in cell competition comes from studies on *scribble* competition in mammalian cell culture (Norman *et al*., 2012). The stress-activated kinase p38 signalling was elevated in the mutant loser cells and was required for their elimination. Moreover, another MAPK often activated in response to stress, the c-Jun N-terminal kinase (JNK), was reportedly up-regulated in *Drosophila* imaginal discs that lost Mahjong or *lgl*, and in *Minute*$^{+/-}$ discs (Tamori *et al*. 2010). This elevation was observed even in absence of competition, suggesting that detection of stress may be an early event in competition, identifying cells as potential losers.

Considering the compelling evidence for the involvement of stress in cell competition, it would be interesting to test whether increased cellular stress is a common characteristic of all loser cells and whether stress also plays a role in super-competition.

1.3.2. Recognition of winner and loser cells

The central rule of cell competition is that two cell populations behave differently in homotypic tissues, then when juxtaposed. This implies that the two cell populations are able to sense one another. This recognition could be mediated either by different secretory signatures, a difference in the composition of cell membranes, or possibly by differences in mechanical properties of the cells, such as cell stiffness. Although the identification of molecular markers of loser and winner cells is by no means complete, some progress has been made. Flower is a transmembrane protein first identified as a calcium channel (Yao *et al*. 2009). In *Drosophila* it has three splicing isoforms. These three isoforms were differentially expressed between winner and loser cells in *Minute* and dMyc competition in wing and eye imaginal discs (Rhiner *et al*. 2010). While Flower^{Ubi} was the dominant protein in homotypic tissues and in competing winner cells, it was down-regulated in competition loser cells. On the contrary, Flower^{LoseA} or Flower^{LoseB} were expressed selectively in loser cells. Their expression was both necessary and sufficient for competition (Rhiner *et al*. 2010). Similarly, knock-down of Flower^{Ubi} was sufficient to drive the elimination of loser cells. In addition to a function in wing imaginal discs, the "Flower code" is sufficient for recognition and elimination of supernumerary postmitotic neurons during retina development in *Drosophila* eye (Merino *et al*. 2013).

The same microarray screen identified another marker of loser cells (Portela *et al*. 2010). Sparc is a secreted protein involved in remodelling of the extracellular matrix and selectively upregulated in competing loser cells. Down-regulation of Sparc in the loser cells accelerated their elimination. Overexpression of Sparc had a protective effect, suggesting that expression

of Sparc is a self-reservation mechanism that could prevent premature elimination of cells experiencing transient difficulties (Portela *et al*. 2010). Sparc was reported to be a marker for a broad range of loser cells, including low-Myc and *lgl*-deficient clones (Portela *et al*. 2010), however others have failed to reproduce these results (Rodrigues *et al*. 2012).

1.3.3. Mechanisms of elimination of out-competed cells

The main questions pertaining to the elimination of loser cells include: (1) What mechanism and pathways are engaged in the losers and in in the winners to affect the elimination of loser cells? (2) What is the mode of elimination of the loser cells? While apoptosis is most common, other mechanisms, e.g. senescence (Bondar & Medzhitov 2010), live extrusion (Hogan *et al*. 2009; Kajita *et al*. 2010), and displacement from a stem cell niche (Jin *et al*. 2008) or displacement and differentiation (Singh *et al*. 2016) have also been reported. (3) How are losers cleared from tissues, e.g. by extrusion or engulfment?

1.3.3.1. Death signalling in loser cells

In the archetypal *Minute* and Myc *Drosophila* imaginal disc models of cell competition loser cells undergo apoptosis (Moreno *et al*. 2002; de la Cova *et al*. 2004). Elimination by apoptosis is conserved in adult *Drosophila* (Kolahgar *et al*. 2015) and in mammals, including Myc competition in the heart (Villa Del Campo *et al*. 2014) and in mouse embryos (Clavería *et al*. 2013; Sancho *et al*. 2013). Death by apoptosis is also detected in other Myc-related competition models, including Ras competition in *Drosophila* discs (Karim & Rubin 1998; Prober & Edgar 2000), BMP competition in imaginal discs (Burke & Basler 1996) and in mouse embryos (Sancho *et al*. 2013) and in Yorkie (Yap) competition (Tyler *et al*. 2007; Ziosi *et al*. 2010). Loser cells undergo apoptosis in Crumbs competition (Hafezi *et al*. 2012), Wingless pathway competition (Vincent *et al*. 2011), JAK/STAT competition

(Rodrigues *et al*. 2012) and in several polarity-factor-related competition models (Brumby & Richardson 2003).

A number of signalling pathways may trigger apoptosis of loser cells. These include Azot and stress-associated pathways such as JNK and NF-κB. Among those, JNK was the most thoroughly studied, but also the most debated. One group reported activation of JNK in *Minute* and dMyc competition losers, stating that inhibition of JNK within the loser clones rescued them from elimination from *Drosophila* imaginal discs (Moreno *et al*. 2002; Moreno & Basler 2004). However, when two other groups globally blocked JNK signalling in *Drosophila* larvae, they failed to rescue *Minute* and dMyc competition losers (de la Cova *et al*. 2004; Tyler *et al*. 2007). JNK signalling has also not been observed in JAK/STAT competition (Rodrigues *et al*. 2012). By contrast, multiple reports indicate that JNK significantly contributes to the elimination of polarity-deficient losers from *Drosophila* epithelia (Brumby & Richardson 2003; Uhlirova *et al*. 2005; Igaki *et al*. 2006; Menéndez *et al*. 2010; Tamori *et al*. 2010; Chen *et al*. 2012). Inhibition of JNK signalling in the loser cells also protected normal cells from out-competition by APC-mutant super-competitors in *Drosophila* gut (Suijkerbuijk *et al*. 2016).

Another protein that reportedly triggers competition-induced death in *Drosophila* is Azot (Merino *et al*. 2015). Azot is a cytosolic, calcium binding molecule that is upregulated in the loser cells specifically during competition. Inhibition of Azot prevented elimination of loser cells in *Minute*, dMyc, JAK-STAT and Wingless competition, but does not affect competition triggered by polarity defects (Merino *et al*. 2015).

An additional pathway has recently been implicated in the elimination of loser cells. Meyer and colleagues (2014) proposed an interesting models in which death of loser cells is triggered by components of the innate immune response, traditionally activated in response to non-self. Toll-related receptors (TRRs) are required to trigger NFκB-dependent apoptosis in dMyc and *Minute* competition in *Drosophila*. Interestingly, different TRRs and

NFκB homologues are involved in dMyc competition (TRRs 2, 3, 8 and 9 activating NFκB homologue *Rel*) than in *Minute* competition (TRRs 3 and 9 activating *Dl* and *Dif*). This in turn results in induction of different pro-apoptotic factors, namely Hid for Myc competition and Reaper for *Minute* competition (Meyer *et al*. 2014).

Since JNK, Azot and NFκB are all reportedly required for *Minute* and Myc competition, further studies are required to clarify their respective contributions and potential interactions.

1.3.3.2. Clearing of loser cells

A number of mechanisms have been suggested for the clearance of dying loser cells. For example, in *Drosophila,* winner cells reportedly engulf losers in both *Minute* and *scribble* competition (Li & Baker 2007; Ohsawa *et al*. 2011). Interestingly, both studies reported that engulfment did not simply follow cell death, but rather that it was an active process necessary for the elimination of loser cells, as mutations in several members of the engulfment machinery blocked cell competition. However, it is also plausible that inhibition of engulfment affected cell competition indirectly, e.g. by affecting cell fitness. Debris of *Minute*$^{+/-}$ cells were also found in the wild-type neighbours during competition between mouse pluripotent cells (Clavería *et al*. 2013; Sancho *et al*. 2013). In contrast, Lolo and colleagues (2012) did not confirm the requirement of engulfment for the elimination of loser cells in *Minute*, polarity-related and dMyc competition in *Drosophila*. Instead, they and others reported that loser cells were extruded from the epithelia and that their debris was taken up by hemocytes, attracted by loser cells which secreted tyrosyl tRNA synthetase downstream of JNK signalling (Lolo *et al*. 2012; Casas-Tintó *et al*. 2015).

Another possible fate of loser cells is extrusion from the epithelial monolayer. Extrusion was reported for *lgl*/Mahjong in *Drosophila* and in canine MDCK cells (Tamori *et al*. 2010) and for *scribble* competition and Yap

competition in MDCK cells (Norman *et al*. 2012; Chiba *et al*. 2016). Interestingly, extrusion of MDCK cells transformed with Ras or Src was not preceded by cell death (Hogan *et al*. 2009; Kajita *et al*. 2010), suggesting that extrusion, in addition to removing cell debris, may serve as an independent way of eliminating loser cells.

1.3.4. The range of cell competition

It remains unclear whether, and in what types of competition, direct cell-cell contact in required, but by general consensus cell competition is held to be a short range phenomenon. Early on it has been shown that in *Drosophila* imaginal discs wild-type cells grow faster in the proximity to *Minute*$^{+/-}$ clones, than they do further away (Simpson & Morata 1981). In this study labelled secondary clones were induced within wild-type regions of mosaic wing imaginal discs. These secondary clones were significantly larger near the wild-type/*Minute*$^{+/-}$ borders than in the centre of wild-type regions. Correspondingly, in many models of cell competition loser cell elimination occurs mainly near the border with winner clones. For example, apoptotic cells were primarily found at the borders of *Minute*$^{+/-}$ clones in *Drosophila* imaginal discs (Li & Baker 2007) and in adult *Drosophila* intestine (Kolahgar *et al*. 2015) and near the border of *lgl* looser clones (Tamori *et al*., 2010). Similarly, apoptosis of host hepatocytes occurred within one or two cell diameter distance from transplanted foetal hepatocytes in mouse liver (Oertel *et al*. 2006). Together, this data suggest but does not prove that direct cell-cell contact is required for the elimination of loser cells. Another line of evidence comes from studies of the "Flower code" (Rhiner *et al*. 2010). If loser cells are to be recognised by the isoforms of the Flower protein presented at their surface, than direct contact with the winner cells is be required.

The range of Myc competition varies between different models. In adult mouse heart apoptosis was 17-fold more frequent in wild-type cardiomyocytes in direct contact with Myc-overexpressing cardiomyocytes

than in those not contacting the mutant cardiomyocytes (Villa Del Campo *et al*. 2014). Apoptosis was also detected mainly in proximity of clone border in dMyc competition during *Drosophila* development, but in this case the loser cells sensed winners in a range of about 10 cell diameters (de la Cova *et al*. 2004). What is more, experiments on the *Drosophila* S2 cells showed that soluble factors can mediate Myc competition even in absence of cell-cell contact. Incubation of wild-type or mutant S2 cells in conditioned medium from competitive co-cultures reproduced the effects of cell competition by inducing growth of high-dMyc cells and triggering apoptosis of the wild-type cells (Senoo-Matsuda & Johnston 2007). Incubation of wild-type cells in a medium conditioned by mutant cells (or vice-versa) had no such effect, confirming that competition requires communication between two cell populations. However, if the medium had been conditioned by one pure population and then by the other (in whichever order) and then transferred on naïve wild-type or mutant cells, death of the first and proliferation of the second was observed. This suggests that both the initial recognition and the effector phase of dMyc competition were mediated by soluble factors (Senoo-Matsuda & Johnston 2007). Similar results were obtained in mouse embryonic cell culture, while studying the Myc-dependent BMP competition. The cells were cultured in a trans-well system where BMP-deficient cells shared medium with normal cells and this was sufficient to reduce numbers of the mutant cells (Sancho *et al*. 2013).

1.4. **Prevalence of cell competition**

Cell competition was first discovered in imaginal discs of *Drosophila* larvae (Morata & Ripoll 1975). These simple, flat epithelia remained for many years the predominant system to study cell competition. However, this posed the question of how prevalent is cell competition in other tissues and organisms, and at other developmental stages. Already the earliest studies indicated that cell competition may not occur in all tissues or under all conditions. The levels of competition can even differ between various regions of the same

compartment. For instance *Minute*$^{+/-}$ clones are eliminated earlier in the centre than in the periphery of a wing imaginal disc (Simpson 1979). Moreover, during *Drosophila* development cell competition is strong in some tissues, while weak or absent in others. In an early paper Simpson (1981) showed that competition is particularly pronounced in wing imaginal discs, but not observed between histoblasts. Similarly, there are restrains on when competition does occur. For instance, *Minute* competition in *Drosophila* wing discs ceases at the end of larval development (Simpson & Morata 1981).

Recently, the prevalence of cell competition has been a major focus of the field. The key questions are: (1) Does competition occur in adult tissues? (2) Does competition occur in mammals? (3) Is competition involved in tumorigenesis? (4) In what tissues does competition occur? Although there are no comprehensive studies addressing this last question, in recent years competition has been detected in a number of non-epithelial tissues, including the mouse myocardium (Villa Del Campo *et al*. 2014), the NIH3T3 fibroblasts (Mamada *et al*. 2015) and mouse blastocysts (Clavería *et al*. 2013; Sancho *et al*. 2013).

1.4.1. Cell competition during mammalian development

For years competition has been mainly studied in *Drosophila*, where a number of pertinent mutations and signalling pathways were discovered. Thus, when the field finally expanded to include mammalian systems, it had to be asked how well the molecular mechanisms identified in *Drosophila* are conserved in mammals. Since competition has been mostly studied in development, developing mammals were the most likely candidates to look for the answers.

1.4.1.1. *Minute* competition during mammalian development

The discovery of *Minute* competition in mice provided an early evidence that the mechanisms of cell competition may be at least partially conserved between *Drosophila* and mammals. A heterozygous mutation in the ribosomal protein L24 confers the Belly spot and tail (*Bst*) phenotype in mouse. These mouse *Minute*s differ from their *Drosophila* equivalents in that mammalian development cannot be prolonged. Instead, the mice show decreased pigmentation, abnormal retinal development and characteristic kinked tails. Oliver and colleagues (2004) generated chimeric mice by injecting labelled wild-type cells, or control *Bst* cells, into *Bst* blastocysts. If *Minute* competition were to occur, the contribution of wild-type cells towards adult tissues should be much higher than that of the control *Bst* cells. Indeed, the injection of ten embryonic stem cells per blastocyst resulted in a 50% contribution to the coat for wild-type cells, compared to 5% for the control, suggesting that cell competition occurred at some stage of development. Like in *Drosophila Minute* competition, the size and shape of tissues were preserved. Also like in *Drosophila*, the contribution of wild-type cells varied between tissues, possibly as a result of differing strength of competition (Oliver *et al*. 2004). As a caveat, it should be noted that, although cell competition is a likely explanation for the strikingly high contribution of wild-type cells to certain tissues, no experiments were carried to verify if Bst cells were actively eliminated during development. It is therefore possible that wild-type cells propagated merely due to their cell-autonomously higher proliferation rates.

1.4.1.2. cMyc competition in mouse embryos

cMyc competition during mammalian development has been more extensively studied that *Minute* competition. Claveria and colleagues (2013) induced genetic mosaics in mouse epiblasts, confronting clones with different levels of cMyc, ranging from null to over-expression. All mutants were viable in homotypic tissues. In mosaics, cells with higher levels of cMyc

propagated, while cells with relatively lower levels of cMyc were eliminated, meeting the criteria of cMyc competition. The process recapitulated *Drosophila* cMyc competition in that the larger the difference in cMyc levels, the stronger the competition, and in that it depended on apoptosis of loser cells. Moreover, like in *Drosophila*, competition did not disrupt the size or shape of the developing organs (Clavería *et al*. 2013). The authors also provided and interesting insight into the prevalence of cell competition by showing that the elimination of the loser cells was not continuous, but that it took place in a short time window early in the embryogenesis.

Cell competition has been mainly studied in artificially generated genetic mosaics, which do not give an insight into the existence of endogenous, spontaneous competition. Claveria and colleagues (2013) addressed this issue by studying the distribution of endogenous cMyc in the early mouse embryo. They showed that in the time window when competition was expected to occur, the levels of endogenous cMyc varied from cell to cell in an apparently random distribution. Moreover, cMyc levels were on average twice lower in early apoptotic cells than in non-apoptotic cells (Clavería *et al*. 2013). This suggests that cMyc competition is part of normal development of an early embryo.

Sancho and colleagues (2013) also studied the fate of malfunctioning cells in mouse embryos. In a combination of *in vivo* and *in vitro* assays they showed that cells with defective BMP signalling or defective autophagy, as well as tetraploid cells, are eliminated by apoptosis by wild-type cells. *In vitro*, this elimination depended on establishing different levels of cMyc (Sancho *et al*. 2013). Moreover, in concurrence with Claveria *et al*. (2013), they reported heterogeneous expression of cMyc in the early embryo, and lower levels of cMyc in dying cells.

1.4.2. Cell competition in adult *Drosophila*

Cell competition has been mainly studied during development. However, recent years brought reports on competition in adult tissues, both in *Drosophila* and in mammals. Cell competition could play a dual role in adult organisms, either by promoting tissue fitness by eliminating sub-optimal cells, or by propagating mutant super-competitors.

1.4.2.1. Competition promotes fitness

An example of competition as a homeostatic process, resulting in the elimination of malfunctioning loser cells, is *Minute* competition in the posterior midgut of adult *Drosophila* (Kolahgar *et al*. 2015). Our group showed that normal cells induce apoptosis and delamination of *Minute*$^{+/-}$ cells, targeting both stem and differentiated cells (Kolahgar *et al*. 2015). An important characteristic of *Minute* competition in *Drosophila* imaginal discs is the compensatory proliferation of wild-type cells, which helps to replace the eliminated loser cells with a heathy tissue. A similar phenomenon was reported in the adult *Drosophila* gut. In this system, *Minute* mutations cell-autonomously activated JNK stress signalling at sub-lethal levels. This lead to secretion of the cytokine Unpaired-3 by the loser cells. Unpaired-3 in turn activated JAK-STAT signalling in the neighbouring normal cells, promoting their proliferation and self-renewal (Kolahgar *et al*. 2015).

Tamori and Deng (2013) reported an interesting example of competition in a homeostatic tissue that is no longer dividing. In post-mitotic *Drosophila* ovary follicular epithelia *Minute*$^{+/-}$ or Mahjong-deficient cells are viable in homotypic tissues, but undergo apoptosis when juxtaposed with wild-type cells. Like in other competition models, the size and shape of the follicles was preserved, but instead of compensatory proliferation, the wild-type cells filled the vacated space by undergoing cellular hypertrophy by additional endocycling. Unlike compensatory proliferation, compensatory hypertrophy did not require JNK signalling (Tamori & Deng 2013).

Interestingly, mutations in dMyc, Yorkie, *lgl* and *scribble* did not trigger competition either in proliferating or post-mitotic follicular epithelia.

Another example of cell competition in an adult *Drosophila* ovary comes from studies on the ovarian germ line stem cells (GSCs). Differentiation-deficient (*bam*- and *bgcn*-mutant) GSCs out-compete normal ones. They do so not by enforcing apoptosis or differentiation, but by invading their space and gradually pushing them out of the niche (Jin *et al*. 2008). This particular model of competition does not depend on dMyc signalling. Nevertheless, a gradient of dMyc signalling can induce competition between GSCs, with higher levels of dMyc granting a competitive advantage (Rhiner *et al*. 2009). In addition, the authors observed higher dMyc levels in stem cells than in their progeny and suggested that the progeny is continuously out-competed for niche-secreted Dpp, which leads to the initiation of differentiation process.

1.4.2.2. Competition promotes tumorigenesis

A recent study from our group provides an example of a tumorigenic function of cell competition in an adult tissue. When Suijkerbuijk and colleagues induced $APC^{-/-}$ clones in *Drosophila* intestines, the resulting adenomas acted as super-competitors, killing the surrounding cells (Suijkerbuijk *et al*. 2016). Blocking cell competition by inhibiting apoptosis protected host tissues from the expansion of adenomas, indicating that cell competition is required for growth of APC-mutant tumours. JNK signalling was required both for cell-autonomous proliferation of the $APC^{-/-}$ clones and for the elimination of wild-type cells. Unlike in the *Minute* competition gut model (Kolahgar *et al*. 2015), proliferation of the winner cells did not depend on Unpaired-3 and the JAK/STAT pathway. Instead, it required activation of the Hippo-pathway target Yorkie. Cell competition required a gradient of Yorkie signalling. Reducing the gradient by increasing activation of Yorkie throughout the epithelium rescued wild-type clones from out-competition without affecting them cell-autonomously.

1.4.2.3. Competition between different developmental lineages

Singh and colleagues (2016) recently provided an interesting example of competition between cells from two different lineages, which occurs in adult *Drosophila* testis. The Mlf1-adaptor molecule (Madm) is a tumour suppressor. In the testis, it is specifically expressed in somatic cyst stem cells (CySCs) and that regulates their competition with germline stem cells for niche occupancy. Madm knock-down in CySCs resulted in paracrine signalling that activated EGF receptor signalling and integrin expression in neighbouring CysSCs, allowing them to over-proliferate and out-compete the germline stem cells. This out-competition occurred not by killing the germline cells, but by displacing them from the niche and by promoting their differentiation. Conversely, over-activation of Madm signalling in CySCs led to their elimination by the germ stem cells and promoted germ stem cell tumour formation (Singh *et al*. 2016).

1.4.3. Cell competition in adult mammals

Cell competition in adult mammals is a relatively new and unexplored field. The earliest example comes from studies on rat liver (Oertel *et al*. 2006). Following partial hepatectomy, foetal liver cells were transplanted into host animals. These more proliferative liver stem/progenitor cells progressively replaced the less proliferative neighbouring host hepatocytes by inducing their apoptosis. The transplanted cells differentiated, forming functional livers of normal size (Oertel *et al*. 2006). Another example on competition that employed re-population of an adult tissue is the p53 competition in mouse bone marrow (Bondar & Medzhitov 2010; Marusyk *et al*. 2010) described in an earlier chapter.

More recently, Villa del Campo and colleagues (2014) induced competition in adult mouse hearts by generating genetic mosaics. When they over-expressed cMyc at levels that did not alter size or anatomy of the heart, the mutant cells gradually replaced the neighbouring wild-type cardiomyocytes.

cMyc competition in the heart resembled other cMyc and *Minute* competition models in a number of ways. Firstly, the strength of competition was proportional to the difference in cMyc levels between loser and winner cells, with the strongest super-competitors possessing the highest levels of Myc. Secondly, consistently with studies on *Minute* competition in *Drosophila* wing discs (Simpson 1981), the strength of competition was not uniform across the heart. Thirdly, the replacement of wild-type cells was phenotypically silent, neither affecting the size nor anatomy of the heart. Finally, the expansion of the mutant cells required death of the wild-type cells. Notably, the mode of cells death differed form dMyc competition in developing *Drosophila*. While apoptosis has been detected in the foetus, the authors suggested autophagic cell death as a major contributor to the elimination of the loser cells in the adult heart (Villa Del Campo *et al*. 2014).

The disadvantage of studying cell competition in adult mammalian tissues is that it often requires time-frames of months (Oertel *et al*. 2006; Bondar & Medzhitov 2010; Villa Del Campo *et al*. 2014) rather than days or weeks. Yet, while other systems proved convenient in unravelling the molecular mechanisms of competition, adult mammalian models may have the greatest potential to yield medical therapies. In particular, they could help to develop practical solutions for regenerative medicine and for cancer therapy.

1.5. **Functions and applications of cell competition**

The primary function of cell competition is thought to be the maintenance of tissue fitness by removing defective cells (Morata & Ripoll 1975). To meet the criteria of cell competition, the loser cell population has to be autonomously viable. Thus, removing the loser cells should not be required for the survival of the tissue, but only to improve its quality. This holds true for the elimination of *Minute*$^{+/-}$ and Myc-deficient cells from normal tissues. However, there are interesting exceptions to this rule, where cell competition not only improves the quality of a tissue, but also assures its

long-term survival. Firstly, a mutation that is viable early on in development may be deleterious at a later stage. Secondly, cell competition can eliminate cells that acquired tumour-promoting mutations before they have a chance to progress into cancer. While the elimination of loser cells by normal cells serves to protect against cancer, super-competition can promote cancer development.

In addition to its physiological roles, cell competition may also have therapeutic uses, in particular by serving as a tool for regenerative medicine.

1.5.1. Cell competition and cancer

Most known models of competition are triggered by mutations in oncogenes or tumour suppressors, strongly suggesting that cells competition may play a role in tumour development. There are two ways in which competition could be involved in cancer: (1) Mutant cells could be recognised as malfunctioning, and eliminated as such. In this scenario, cell competition would play a protective, homeostatic role. (2) Certain mutations could increase cell fitness, enabling them to colonise tissues at the expense of normal cells. An increasing body of evidence supports both pro- and anti-tumour roles of cell competition.

1.5.1.1. Super-competitors and cancer

The possibility that cell competition promotes cancer was first suggested when it was discovered that cells over-expressing the oncogene dMyc are able to out-compete wild-type cells (Moreno & Basler 2004; de la Cova *et al*. 2004). Since then, mutations in other tumour-associated genes were showed to create super-competitors. These include mutations in members of Wnt and Hippo pathways, and in p53, which is the most commonly dysregulated protein in cancer.

The most compelling evidence for a role of super-competition in tumorigenesis comes from two recent studies in *Drosophila*. As described in a previous section, growth of $APC^{-/-}$ adenomas in healthy adult *Drosophila* guts requires cell competition (Suijkerbuijk *et al*. 2016). Another study in *Drosophila* (Eichenlaub *et al*. 2016) provides insight into the role of cell competition in the formation of metastatic tumours. Over-expression of EGFR in imaginal wing discs caused benign hyperplasia. Over-expression of the micro-RNA miR-8 caused a modest reduction in disc size. A combination of these two mutations created super-competitors able not only to kill the surrounding cells, but also to form neoplastic growths and to metastasize. The neoplastic transformation and metastasis depended on the ability of the double-mutant cells to induce apoptosis and to engulf nearby cells. The engulfment was required for giant polyploid cells to form within the mutant cell population. Suppression of engulfment did not protect the surrounding cells from apoptosis, but it prevented neoplastic transformation and metastasis. This suggests that cell competition played a dual role in tumour progression by propagating mutant cells within the epithelium and by promoting transformation into metastatic cells. Interestingly, the mutant cell population was heterogeneous, with both small and giant polyploid cells, and inhibition of apoptosis within the mutant clones prevented the formation of giant cells, neoplasia and metastasis (Eichenlaub *et al*. 2016). This suggests that competition occurred both within the mutant clones, and between mutant and wild-type cells.

In addition to its role in the formation and progression of primary tumours, it was suggested that cell competition facilitates colonising of secondary tumour sites (reviewed by Wagstaff *et al*. 2013). For instance, it has been reported that when gastrointestinal cancer cells form secondary tumours in the abdominal cavity, they rely on their ability to kill mesothelial cells by apoptosis. *In vitro*, death of the mesothelial cells required direct cell-cell contact and partially dependent on the pro-apoptotic Fas/Fas ligand interaction (Heath *et al*. 2004). Consistently, in a mouse model, colonisation

of the liver by Fas-expressing tumour cells was blocked when apoptosis was suppressed in the host tissues (Li *et al*. 2009).

1.5.1.2. Cell competition in the elimination of tumour cells

Several tumour-promoting mutations are known to turn cells into competitive losers, suggesting that cell competition plays a role in the elimination of transformed cells before they had a chance to accumulate further mutations and progress into cancer. Mutations in the polarity factors, described in a previous section, are a good example of this protective mechanism.

A compelling evidence for a tumour-protective function of cell competition has been recently provided by Martins *et al*. (2014). T-cell progenitor cells in the thymus are genetically unstable and therefore prone to undergo malignant transformation. To prevent accumulation of harmful mutations, T-cell progenitors are continuously replaced by younger cells arriving from the bone marrow. Martins *et al*. (2014) reported that the thymus-residing progenitors persisted for much longer when fresh progenitors were not provided from the bone marrow. The fact that the resident cell population was lost in the presence, but not in the absence of another cells population suggests cell competition. Consistently, inhibiting Interleukin-7 signalling in the bone-marrow-derived progenitors, and thus reducing their fitness level, rescued the resident cells. Martins and colleagues (2014) further reported that cell competition was necessary to prevent T-lineage acute lymphoblastic leukaemias. When a thymus was grafted into an animal that could not supplement fresh progenitor cells, most animas developed leukaemias. The disease was prevented by reconstituting the bone marrow, but only if it occurred early after the transplantation. This suggests that, at least in the thymus, cell competition prevents the initiation of cancer development, rather that eliminating irreversibly transformed cells. The authors further proposed that this type of cell competition is triggered by natural differences in the expression programs executed by the “old” and

"new" progenitor cells, rather than by abnormal mutations (Martins *et al*. 2014).

1.5.2. Cell competition and regenerative medicine

Two features of cell competition make it a desirable tool for regenerative medicine. Firstly, even a small number of cells can ultimately take over a tissue. Secondly, many types of competition are phenotypically silent in that they do not affect the anatomy of an organ. Together, this means that, given sufficient time, even a small number of healthy winner cells could revitalise and restore a malfunctioning organ. These healthy cells could be delivered by transplantation, as demonstrated by Oertel *et al*. (2006) and Bondar and Medzhitov (2010). Alternatively, a selected cell population could be given a competitive advantage *in vitro* by chemical or genetic intervention.

Understanding the molecular mechanism controlling which cells propagate and which are eliminated could provide invaluable control over the composition of tissues. A current struggle in the field lays in that those mutations that turn cells into winners, such as loss of p53 (Bondar & Medzhitov 2010) or over-activation of cMyc (Villa Del Campo *et al*. 2014), simultaneously promote tumorigenesis. Hopefully, better understanding of the mechanisms of cell competition will soon overcome these obstacles and enable the first viable competition-based therapies.

1.6. Cell culture models of cell competition

Most work on cell competition in *Drosophila melanogaster* has been carried *in vivo*. The only cells studied *in vitro* are the S2 cells, an immortalised cell line with epithelial morphology, derived from *Drosophila* embryos. S2 cells were used to model dMyc competition and to study the requirement of direct cell contact *versus* secretory factors for the elimination of loser cells (Senoo-Matsuda & Johnston 2007; de la Cova *et al*. 2004).

In contrast to *Drosophila* cell culture, mammalian cell culture recently became a major tool to study cell competition. In particular, cell competition has been extensively studied on the Madin-Darby Canine Kidney epithelial cells (MDCK). MDCK cells were derived from a kidney of a normal adult female cocker spaniel, are hyperdiploid and serve as a canonical line to study mammalian epithelia. In 2D culture, MDCK cells are cuboidal in shape and form tight epithelial monolayers. In co-culture experiments, there is little cell shuffling and individual clones do no mix. Hence, individual clones can be easily distinguished, closely resembling mosaic *Drosophila* imaginal discs. MDCK competition models include Mahjong (Tamori *et al*. 2010) and *scribble* (Norman *et al*. 2012) competition. According to the broad definition of cell competition presented in this overview, apical extrusion of MDCK cells over-expressing Yap (Chiba *et al*. 2016), MDCK cells expressing constitutively active Ras (Hogan *et al*. 2009) and Src-transformed MDCK cells (Kajita *et al*. 2010) by wild-type cells cell can be also classified as competition models. In addition to the above models where a defined mutation triggered competition, a recent study reported spontaneous competitions between sub-clones of MDCK cells (Penzo-Méndez *et al*. 2015). The authors further proposed that cell competition is a common feature of immortalised mammalian cells, as it has been also observed between sub-clones of transformed U2OS human osteosarcoma cells and among non-transformed NIH-3T3 murine embryo fibroblasts. Cell competition between NIH-3T3 has been also reported in another recent study, where cells with reduced Tead (interactor of Yap) become losers, while over-activation of Tead or Myc created super-competitors (Mamada *et al*. 2015). In addition to the above cell culture models, the recent interest in competition in mammalian embryos brought embryonic stem cell culture into the field. For instance Claviera and colleagues (2013) used this system to model Myc super-competition and to address the role of BMP in competition.

Tissue culture provides several advantages over *in vivo* studies in mammals in addition to circumventing concerns over animal welfare. These include the tightly and easily regulated conditions of the culture. Cell culture also provides a convenient platform for molecular studies, an advantage that I exploited in my project. Arguably, one of the main benefits on tissue culture competition models it their suitability for live imaging, a technique on which I have heavily relied in my studies. Live imaging allows one to follow the fate of individual clones and hence to answer questions such as: (1) What is the range of competition? Does it require direct cell contact? Do only cells at the very edge of the clones die? (2) What is the sequence of events that leads to the elimination of cells? Does death follow or precede cell extrusion? Indeed, it was the use or live imaging that allowed us to discover mechanical cell competition in the MDCK system (described later; Wagstaff *et al*., 2016), which was a major focus of my project.

1.7. *scribble*-induced cell competition

1.7.1. *scribble* as a polarity factor and a tumour suppressor

scribble was first discovered in *Drosophila*, where it forms a complex with *Dlg* and *Lgl*. In *Drosophila* epithelia Scribble is localised basally to the adherens junctions, at the basolateral junctions, while in mammalian epithelia, its localisation overlaps with adherens junctions and extends basally (reviewed by Humbert *et al*. 2008). Scribble is a large, multi-domain protein of the LAP (leucine-rich repeats and PDZ) family scaffold protein. In *Drosophila* epithelia, it is required for apico-basal polarity. Larvae lacking functional *scribble* initially develop normally, but are unable to initiate pupal development. As maternal supplies of *scribble* in a larvae are depleted, epithelia fail to differentiate, but instead over-proliferate into invasive, multi-layered amorphous masses that identify Scribble as a neoplastic tumour suppressor.

Much less is known about the role of *scribble* in apico-basal polarity in mammalian epithelia, and while some reports supports its role, others argue against it. Knock-down of *scribble* in the kidney epithelial MDCK cells did not affect the apical marker gp135 and cysts grown in 3D culture appeared to be polarised normally (Qin *et al*. 2005; Norman *et al*. 2012). Similar results were reported by Dow *et al*. (2007), who silenced *scribble* in a 3D culture of MCF10A cells. Incongruously, Zhan *et al*. (2008) reported that knock-down of *scribble* mildly affected polarity of MCF10A cells, as visualised by disorganised Golgi and increased number of cells in acini lumen in 3D culture.

In addition to the controversy regarding its role in apico-basal polarity, mammalian *scribble* differs from its *Drosophila* homologue in a number of ways. For instance, while loss of *scribble* causes upregulation of cyclin E and over-proliferation in *Drosophila* (Brumby & Richardson 2003), it shows no such effect in mammals. Zhan *et al*. (2008) reported that *scribble* knock-down had no significant effect on the proliferation of control breast epithelial MCF10A cells in 3D culture. Moreover, our group showed that loss of *scribble* in 2D culture of MDCK cells reduces their proliferation rate (Wagstaff *et al*. 2016). Furthermore, while *Drosophila scribble*-null cells form multi-layered growths, in 2D culture *scribble*-knock-down MDCK cells grow as monolayers (Qin *et al*. 2005; Norman *et al*. 2012; Wagstaff *et al*. 2016).

Despite the differences between *Drosophila* and mammalian Scribble, in both systems multiple lines of evidence (reviewed by Humbert *et al*. 2008) link *scribble*-deficiency with cancer. In mammals, *scribble* is targeted by oncoviral proteins, including HPV E6/7 proteins, and overexpression of *scribble* has been shown to inhibit transformation of rodent epithelial cells by these proteins. Among other evidence, decreased *scribble* expression has been also observed in human colon and breast cancers (Gardiol *et al*. 2006; Navarro *et al*. 2005). A study on tumorigenesis in mice mammary epithelia showed neoplastic growths similar to those observed in *Drosophila*. When transplanted into host animals, normal pluripotent mammary

epithelial cells were able to generate mammary glands in cleared fat pads, while *scribble*-deficient cells formed ducts that filled with multi-layered epithelium, with a number of mice developing tumours (Zhan *et al*. 2008).

1.7.2. *scribble* competition in *Drosophila*

Cell competition between wild-type and *scribble*-null cells was first observed in *Drosophila* larvae. The study was carried on the columnar mono-layered neuroepithelia of eye imaginal discs (Brumby & Richardson 2003), where *scribble*-null clones were induced in an otherwise wild-type tissue. As expected, the mutant cells lost their morphology and became rounded and multi-layered. Levels of cyclin E within the mutant cells were elevated, ectopic proliferation was observed, and many cells lost their ability to differentiate. However, despite this over-proliferation, there was very little *scribble*-null tissue remaining by the time imaginal discs developed into adult eyes. This was due to cell death, as the size of mutant clones increased when apoptosis was blocked with the baculovirus pan-caspase inhibitor, p35. Together, the viability in homotypic tissues combined with elimination from mosaic tissues indicate competition between *scribble*-deficient and normal cells.

Brumby and Richardson (2003) further showed that elimination of *scribble*-null cells depends on JNK signalling within the clones. Overexpression of a dominant-negative version of Basket (*Drosophila* homologue of JNK) selectively within the mutant cells significantly increased the size of *scribble*-null clones. This was indeed a rescue effect, rather than an autonomous effect of JNK, as overexpression of the dominant-negative version of Basket in wild-type clones did not strongly affect clone size or pupal lethality (Brumby & Richardson 2003). The requirement of JNK for the elimination of *scribble*-deficient cells from *Drosophila* epithelia has been corroborated by others (Uhlirova *et al*. 2005; Herz 2006). Igaki and colleagues (2009) later suggested a mechanism by which JNK signalling is activated in *scribble* mutant clones. They observed increased endocytosis

in *scribble* mutant clones. This endocytosis was essential for translocation of Eiger, a *Drosophila* member of the tumour necrosis factor (TNF) superfamily, to endocytic vesicles in the loser cells. The resulting Eiger-JNK signalling in the endosomes triggered apoptosis of *scribble*-deficient cells (Igaki *et al*. 2009).

In a later report Chen and colleagues (2012) further investigated the mechanisms by which competition suppressed the expansion of *scribble*-deficient clones. They reported that in homotypic imaginal discs the mutant cells hyper-activated Yorkie, which drove the growth of the neoplastic masses. On the contrary, in mosaic tissues, Yorkie activity was suppressed, preventing the overgrowth. Ectopic overexpression of Yorkie in *scribble*-deficient cells was sufficient to rescue *scribble*-deficient clones from elimination by competition (Chen *et al*. 2012).

1.7.3. *scribble* competition in MDCK cells

scribble competition was first studied in mammalian tissues by Norman *et al*. (2012). The group stably transfected the canine epithelial MDCK cells with a *scribble* short hairpin RNA, expressed in a tetracycline-inducible manner. Two days after the addition of tetracycline, the levels of *scribble* were reduced by 90%. In competition experiments, *scribble* shRNA cells were labelled with a red fluorescent dye, mixed with normal MDCK cells and followed by time-lapse microscopy. Within 60 hours of the induction of silencing, 45% of the mutant cells died and left the epithelial monolayer, as detected by staining with a fluorescent death marker. The death was not cell autonomous, as labelled *scribble* knock-down cells survived when surrounded by unlabelled *scribble* knock-down cells (Norman *et al*. 2012). Therefore, *scribble* knock-down cells behaved as losers and were eliminated by normal cells by cell competition. The group then identified mitochondrial apoptosis as the main mode of elimination of the loser cells.

Considering that there are a number differences between *Drosophila* and mammalian Scribble, it is not surprising that the mechanism involved in the elimination of the loser cells vary between those two models. JNK, the stress kinase required for the elimination of *scribble* mutants in *Drosophila*, was not phosphorylated, and therefore not activated, in MDCK cells. Neither did the presence of a JNK-specific inhibitor prevent death of the *scribble* knock-down cells (Norman *et al*. 2012). Instead, activating phosphorylation of another MAPK kinase, the p38 stress-activated kinase, was significantly enhanced in clones of *scribble* knock-down cells surrounded by normal cells. Consistently with a role in the elimination of loser cells, chemical inhibition or expression of a dominant-negative form of p38 blocked elimination of the *scribble* knock-down cells (Norman *et al*. 2012).

1.7.4. Mechanical cell competition

Our group attempted to further characterise the mechanisms of *scribble* cell competition in the MDCK system developed by Norman *et al*. (2012). First, Laura Wagstaff and others in our group (Wagstaff *et al*. 2016) showed that direct contact is required for the elimination of *scribble* knock-down cells, as sharing medium in a transwell system was not sufficient to induce death of the mutant cells. Interestingly, contact with normal clones was also not sufficient for their elimination, and only those clones that were surrounded were efficiently out-competed. This prompted our group to investigate what changes corralling by normal cells inflicts on the loser clones.

As discussed before, *scribble* knock-down cells lose their cuboidal morphology, increasing their adhesion surface and assuming a flattened, pancake-like shape (Qin *et al*. 2005; Norman *et al*. 2012; Fig.1.2a-b, Fig.1.3a-f). Using live imaging, Laura Wagstaff observed that in sub-confluent cultures, when growing clones come into contact, clones of *scribble* knock-down cells become compacted, and their density exceeds that of the surrounding normal cells (Fig.1.2.c, Fig.1.3a-f). She then asked whether this enforced compaction is sufficient to eliminate the loser cells.

For this reason, she plated *scribble* cells on their own, at different initial densities. When few cells were plated, they grew to reach a homeostatic density of about 1/3 of that reached by normal cells (Fig.1.3.a-c,f-j). Strikingly, when the mutant cells were plated at a high initial density, their numbers dropped over time, again to reach a homeostatic density of about 1/3 that of normal cells (Fig.1.3.j). This suggests that *scribble* knock-down cells are hypersensitive to compaction and that overcrowding is sufficient to eliminate the excess cells. Further experiment carried out by me and described in the results section show that *scribble* knock-down cells are out-competed by mechanical compaction. **We term the elimination of loser cells by mechanical insults, rather than chemical exchange, mechanical cell competition.**

Mechanical competition has been also recently reported in *Drosophila* pupal

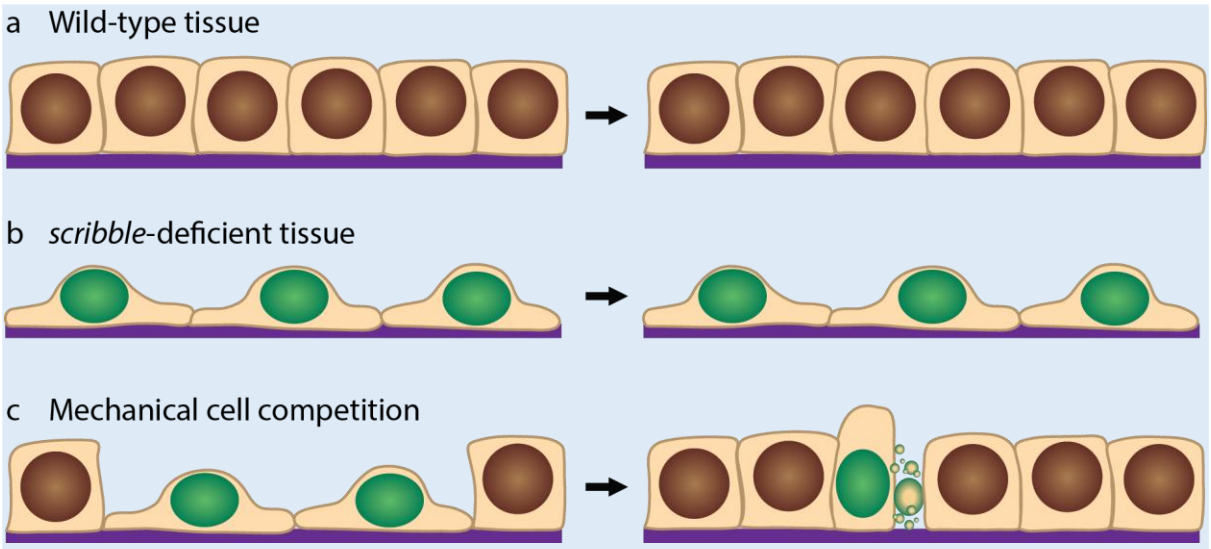


Figure 1.2. Mechanical cell competition.
Schematic representation of *scribble* mechanical competition in MDCK cell culture. Homeostatic cell density in homotypic culture of *scribble*-deficient cells (**b**) is lower than that of wild-type cells (**a**), but the mutants are viable. (**c**) When surrounded by wild-type cells, *scribble*-deficient cells become compacted and die by apoptosis.

midline (Levayer *et al*. 2016). In this case clones of cells expressing and activated form of Ras acted as super-competitors, causing compression of

the surrounding normal tissue and triggering apoptosis in cells up to several cell diameters away from the mutant clones.

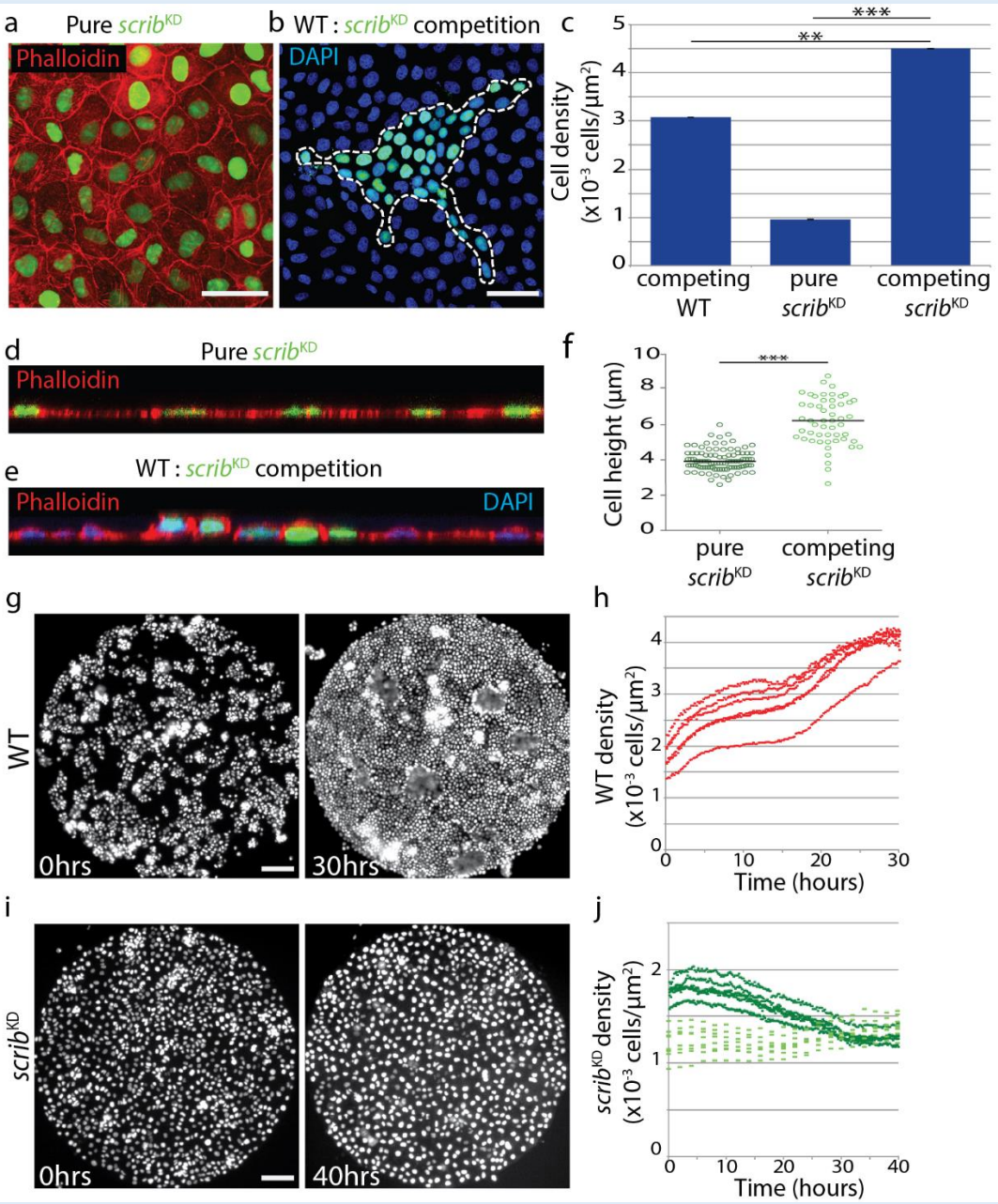


Figure 1.3. Competition boosts cell density in *scrib*^{KD} clones.
From Wagstaff *et al.*, 2016; modified. (**a**) Confluent GFP-labelled *scribble* knock-down (*scrib*^{KD}) cells stained with Phalloidin (filamentous actin). (**b**) Competing unlabelled wild-type (WT) and GFP-labelled *scrib*^{KD} cells counterstained with DAPI. (**c**) Quantification showing average (±s.e.m.) cell density values of pure *scrib*^{KD} cultures and of competing WT and *scrib*^{KD} cells as in **a** and **b**. (**d**,**e**) Confocal xz sections of representative GFP-labelled *scrib*^{KD} cells in pure cultures (**d**) or co-cultured with WT cells (**e**). (**f**) Quantifications of cell height from images as in **d** and **e**. Black bars = median. (**g**,**i**) Representative stills from time lapse imaging of WT (**g**) and scrib^{KD} (**i**) cells growing on micropatterns (800 mm^2). (**h**,**j**) Quantifications of cell density over time from movies as in **g** and **i**. Each dotted line corresponds to a different micropattern. Scale bars, 100 µm (movie sequences) and 50 µm (immunofluorescence images). **P<0.005, ***P<0.0005 by KS test.

1.7.5. Directional cell migration in mechanical competition

During mechanical competition, cell density within loser clones exceeds that of the surrounding wild-type cells, suggesting active compaction (Fig.1.1, Fig.1.2). While investigating how this compaction is brought about, Laura Wagstaff (Wagstaff *et al*. 2016) made an unexpected discovery. She observed that, upon contact, *scribble* knock-down and wild-type clones rapidly engage in a highly directional collective cell migration, with *scribble* knock-down clones always at the migrating front and wild-type cells always at the back (Fig.1.4a). When, instead of confronting a single wild-type clone, *scribble*-deficient clones are corralled by wild-type cells, directional cell migration leads to compaction of the loser cells. While this behaviour may facilitate and accelerate mechanical competition, it is not required for the elimination of mechanical losers. When Laura Wagstaff and others (Wagstaff *et al*. 2016) inhibited directional migration by disrupting E-cadherin junctions, they prevented active cell compaction (Fig.1.4b-d). This resulted in delayed elimination of *scribble* knock-down cells (Fig.1.4e). However, it did not rescue the mutant clones from out-competition. In time, as density of wild-type cells increased due to proliferation, the mutant clones became progressively more crowded, until they were eventually eliminated, suggesting that active compaction promotes, but is not required for mechanical competition. Part of my PhD project dealt with the characterization of the mechanisms underlying this type of directed cell migration.

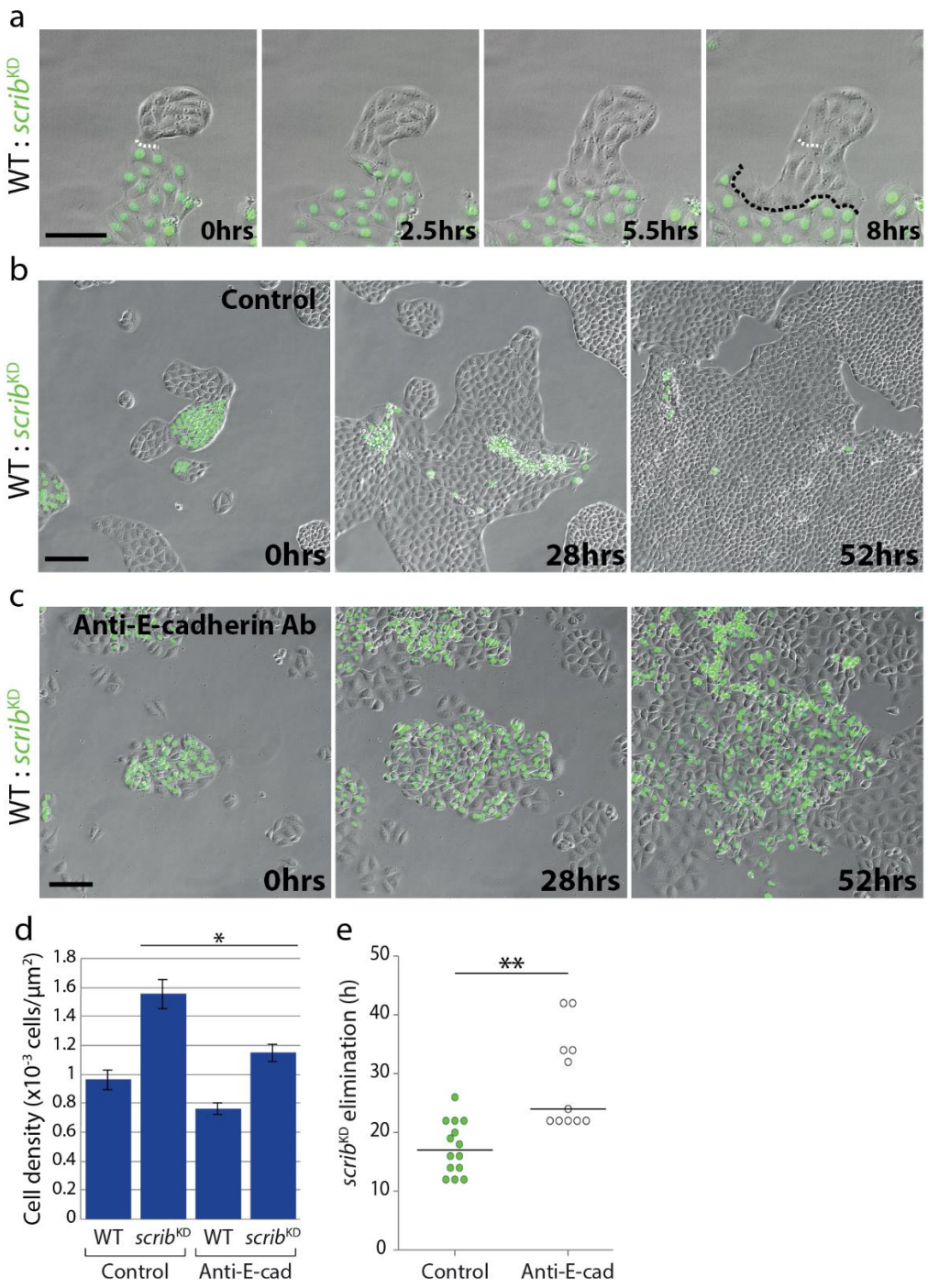


Figure 1.4. Contact-induced migration promotes compaction of *scrib*^{KD} cells.
(**a-c**) Stills from live-imaging. (**a**) Wild-type (WT) and GFP-labelled *scrib*^{KD} clones engage in directional migration upon contact. White dashed line = initial contact point; black dashed line = final contact point (**b**-**e**) Disrupting cell junctions by E-cadherin blocking antibody (Ab) and calcium removal prevents contact-induced migration (**c**), compaction (**d**) and delays competition (**e**) compared with control (**b**). Scale bars, 100 mm. *P<0.05, **P<0.005 by KS test.

Chapter 2. *SCRIBBLE*-DEFICIENT CELLS ARE ELIMINATED BY MECHANICAL COMPACTION

2.1. Introduction

To study the out-competition of *scribble*-deficient cells, I used a cell line generated by Norman and colleagues (2012) that carries *scribble* shRNA (*scrib*^{KD}) inducibly expressed in presence of tetracycline. As discussed in the introduction silencing of *scribble* changes the morphology of MDCK cells. While normal cells (wild-type or *scrib*$^{KD\text{-}TET}$) are cuboidal in shape, the mutants (*scrib*$^{KD+TET}$) flatten, increasing their adhesive surface. This results in a homeostatic density of approximately 1/3 of that of normal cells (Wagstaff *et al*. 2016; Fig.1.2, Fig.1.3). Surprisingly, Laura Wagstaff from our group observed that in co-culture, when surrounded by normal cells, clones of *scrib*$^{KD+TET}$ cells not only match, but exceed the density of the normal cells, suggesting active compaction (Fig.1.2, Fig.1.3). Therefore, we hypothesized that *scrib*$^{KD+TET}$ cells might be hypersensitive to density and that this hypersensitivity is the reason for their elimination when surrounded by normal cells. The first indication that *scrib*$^{KD+TET}$ cells are indeed hypersensitive to density came when Laura Wagstaff used live imaging to follow the numbers of *scrib*$^{KD+TET}$ cells and of normal cells (*scrib*$^{KD\text{-}TET}$) plated in micropatterns, where they formed micro-cultures of a defined, homogenous density and size, convenient for quantitative analysis. This showed that, when *scrib*$^{KD+TET}$ cells were plated at high initial numbers, the density of the culture decreased over time until the homeostatic density was reached (Fig.1.3j). This suggested that the mutant cells are indeed hypersensitive to density and that excess cells are eliminated.

Following on this finding, I decided to directly test whether enforced compaction is sufficient to eliminate *scrib*$^{KD+TET}$ cells. After having shown

that this is indeed the case, I then went on to investigate the mechanisms of elimination of *scrib*$^{KD+TET}$ cell by mechanical means.

2.2. **Forced compaction induces apoptosis of *scribble*-deficient cells**

To test whether enforced compaction is sufficient to eliminate *scrib*$^{KD+TET}$ cells in the absence of a winner cell population I adapted a method previously used to compress MDCK cells (Eisenhoffer *et al*. 2012), where the cells grow on a stretched PDMS substrate, which is then released to induce overcrowding (Fig.2.1a). For my purpose, I cultured either normal or mutant cells at two densities: at confluence and at sub-confluence (Fig.2.1a, left panel). Upon release of the PDMS membranes, the confluent cultures became highly compacted, while cells grown at sub-confluence were left with enough available space, to allow them to retain their flatten morphology (Fig.2.1a, right panel). I then carried immunofluorescent staining against activated Caspase-3 and measured the fraction of dying cells (scored as the number of apoptotic events relatively to the number of intact nuclei per imaged field). The level of death in sub-confluent culture, representing the background level of death, was somewhat higher in *scrib*$^{KD+TET}$ cultures than in wild-type (*scrib*$^{KD-TET}$) cultures (0.39±0.69% for wild-type cells *vs* 3.11±2.63% for mutant cells; Fig.2.1b-d). This was expected, and is typical for *scrib*$^{KD+TET}$ monocultures. Strikingly, while the fraction of dead wild-type cells did not increase significantly with density (0.39±0.69% at low density *vs* 0.52±0.5% at high density; Fig.2.1b,d), it tripled in *scrib*$^{KD+TET}$ cell monocultures (3.11±2.63% at low density *vs* 10.89±3.56% at high density; Fig.2.1c,d), indicating that *scrib*$^{KD+TET}$ cells are hypersensitive to compaction and that compression alone is sufficient to induce death by apoptosis.

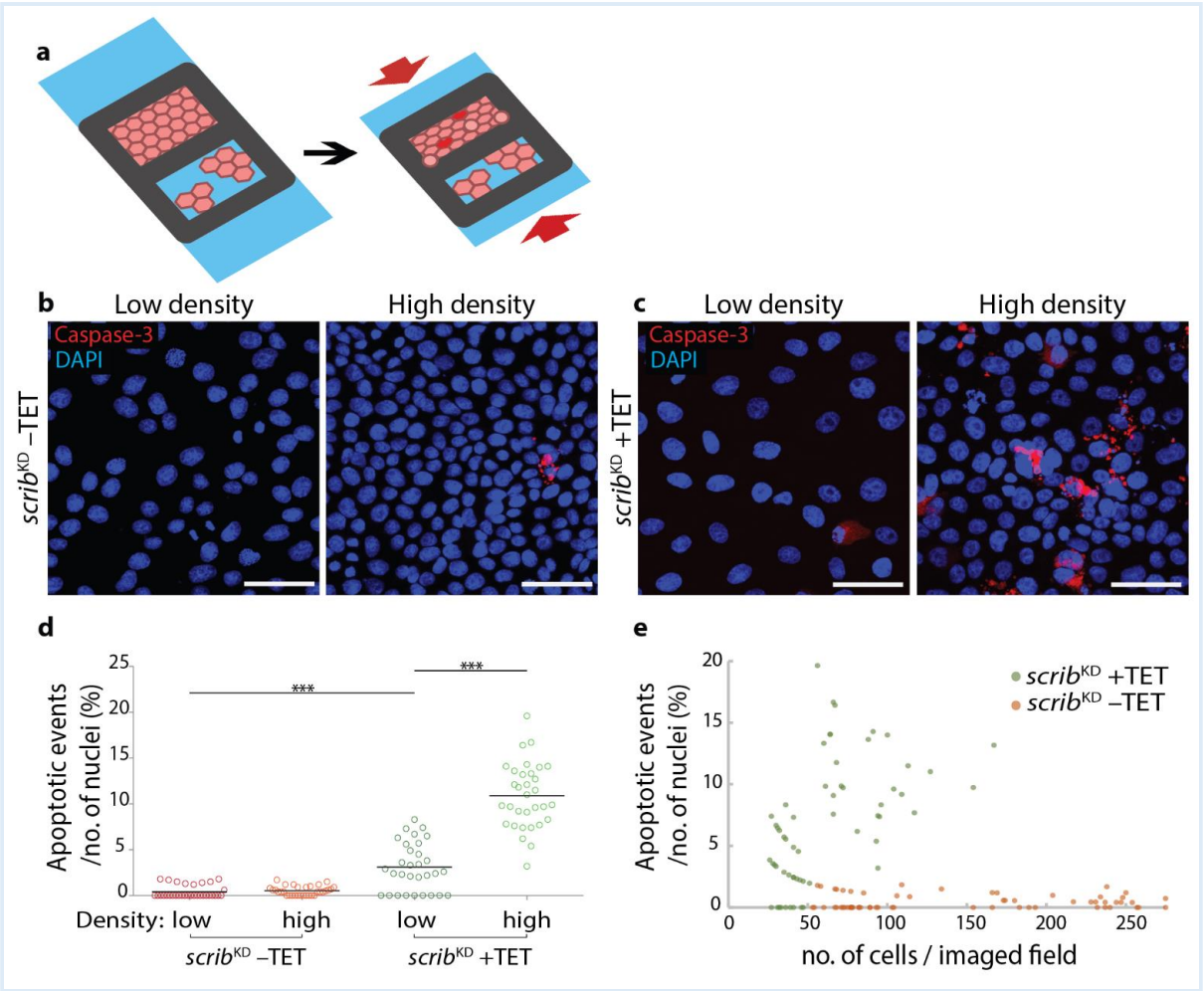


Figure 2.1. Forced compaction induces apoptosis of *scribble*-deficient cells.
(**a**) Experimental design: a PDMS pool (grey) with two rectangular chambers was placed on a stretched PDMS membrane (blue). *scrib*^{KD} cells were cultured in the chambers at two densities: at confluence and at sub-confluence. Upon release of the stretch, only the confluent culture became crowded. Apoptotic cells are shown in red. (**b,c**) Five hours after the release of the membrane the samples were fixed and stained against activated Caspase-3. Representative confocal images of un-compressed (sub-confluent, left panel) and compressed (confluent, right panel) cultures. Scale bars = 50 μm. (**b**) Control cells (*scrib*$^{KD-TET}$). (**c**) *scrib*$^{KD+TET}$ cells were treated with tetracycline for 3 days before fixing. (**d**) Quantification of cell death events (activated Caspase-3) from images as in (**b,c**). Black bars = mean. *** $p<0.0005$ by KS test. (**e**) Alternative way of representing data from (**d**).

Having demonstrated that compression can induce death of *scrib*$^{KD+TET}$ cells, I then set out to confirm that the levels of over-crowding seen in the PDMS-based cell compression assay are comparable to those observed during competition. The homeostatic density of *scrib*$^{KD+TET}$ monocultures, as measured by Laura Wagstaff, was approximately 0.001 cells per μm^2

(Fig.1.1f; equivalent of 40 cells/imaged field in the compression assay). *scrib*$^{KD+TET}$ cells surrounded by wild-type cells reached approximately 0.0045 cells per μm^2 (Fig.1.1.f; or 180 cells/field). Strikingly, compression experiments indicated that increasing the density of *scrib*$^{KD+TET}$ cells to as few as 60 cells/imaged field was already sufficient to trigger apoptosis (Fig.2.1e). Moreover, fields with over 120 mutant cells were only rarely recovered, suggesting that higher densities are not sustainable. Together, these data suggest that *scrib*$^{KD+TET}$ cells die even at lower densities than those reached during competition, and confirm that the levels of compaction applied in the PDMS-based compression experiments were not excessive.

The density of competing wild-type MDCK cells, as measured by Laura Wagstaff, was approximately 0.003 cells per μm^2 (Fig.1.3c,j), which is equivalent to 120 cells/imaged field in the PDMS-based compression assay. The maximal observed density of compressed wild-type cells was approximately 250 cells/field (Fig.2.1e). Yet, even at those extreme densities, I observed no increase in the frequency of cell death (Fig.2.1e). This agrees with previous reports that overcrowding triggers predominantly non-apoptotic extrusion of normal cells (Eisenhoffer *et al*. 2012), suggesting that the response of *scrib*$^{KD+TET}$ cells to compaction is both quantitatively and qualitatively different to that of normal cells, in that apoptosis is the predominant response to overcrowding of mutant, but not wild-type MDCK cells.

2.3. **Known mechanisms of extrusion of MDCK cells are not required for *scribble*-driven competition**

In their original study on *scribble* competition in MDCK cells Norman and colleagues (2012) observed both apoptosis and apical extrusion of out-competed loser cells, and proceeded to characterised their contribution to the elimination of the mutant cells. When juxtaposed with normal cells, many *scrib*$^{KD+TET}$ cells within the monolayer, and all of the apically extruded

losers, stained positive for activated Caspase-3, and had fragmented nuclei. This suggests that apoptosis is the predominant mode of elimination of losers, and also that death may occur before extrusion. Consistently, when the co-cultures were treated with the Myosin-II inhibitor Blebbistatin, which blocked apical extrusion of apoptotic cells, the number of Caspase-3-positive *scrib*$^{KD+TET}$ cells surrounded by normal cells massively increased, suggesting that apoptosis of the losers occurred independently of apical extrusion. Results of the PDMS-based cell compression assays described in the previous section further support the principal role of apoptosis in the elimination of *scrib*$^{KD+TET}$ cells. However, following on the observation by Eisenhoffer and colleagues (2012) that normal MDCK cells respond to over-crowding by live cell extrusion, I decided to further investigate the role of cell extrusion in competition.

The Rosenblatt group recently characterised the molecular mechanism of extrusion of living and apoptotic MDCK cells (Eisenhoffer *et al*. 2012; Gu *et al*. 2011). Gu and colleagues (2011) observed that apoptotic MDCK cells secreted the bioactive lipid, sphingosine-1-phosphate (S1P). S1P then bound to S1P receptor 2 (S1P2) on the surrounding non-apoptotic cells, triggering Rho signalling. Activation of Rho in turn led to formation of an acto-Myosin ring around an apoptotic cell, and ultimately to contraction of the ring, which pushed the apoptotic cell out of the epithelium. A similar mechanism was also responsible for the elimination of supernumerary cells during forced compaction. In this case, activation of the stretch-activated-channel Piezo1 led to production and secretion of S1P and to apical extrusion of living cells (Eisenhoffer *et al*. 2012).

To test whether live extrusion plays a role in the elimination of *scrib*$^{KD+TET}$ cells, I treated competing co-cultures with the Piezo1 inhibitor Gd^{3+} (Fig.2.2a). Gd^{3+} reportedly prevents live extrusion of MDCK cells in the PDMS-membrane compression experiment (Eisenhoffer *et al*. 2012). However, it had no appreciable effect on the elimination of *scrib*$^{KD+TET}$ cells

by wild-type cells in competition assays, as observed by live imaging (Fig.2.2a). Similarly, the downstream extrusion pathway, common for the elimination of both apoptotic and non-apoptotic MDCK cells, did not seem

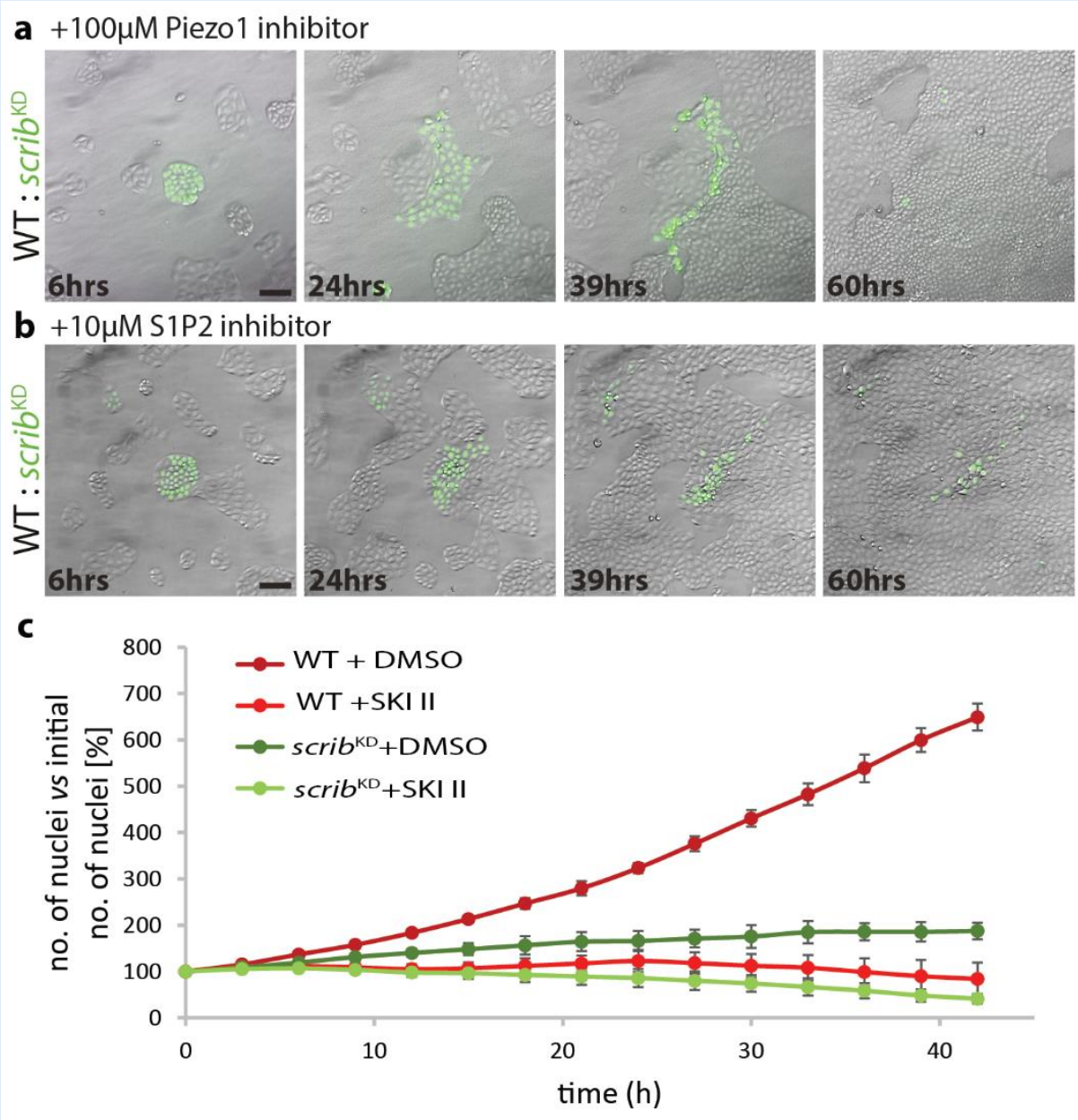


Figure 2.2. Neither S1P2 nor Piezo1 are required for elimination of *scribble*-deficient cells.

(**a**,**b**) Stills from time-lapse movies of WT and *scrib*^{KD} cells co-cultured in the presence of tetracycline and either a S1P2 inhibitor (JTE013, **a**) or Piezo1 inhibitor (Gd^{3+}, **b**). Scale bars=100 µm. (**c**) Quantification of cell number in monocultures of WT and *scrib*^{KD} cells grown in the presence of tetracycline and either DMSO or the sphingosine kinase inhibitor SKI II.

to be involved in mechanical cell competition, as inhibition of S1P2 with the JTE-013 inhibitor at a dose previously used by the Rosenblatt group (Eisenhoffer *et al*. 2012; Gu *et al*. 2011) failed to affect compression and elimination of *scrib*$^{KD+TET}$ cells (Fig.2.2b).

I then investigated the involvement of S1P, a common effector of the live extrusion pathway and of the extrusion of apoptotic cells. Unfortunately, in the long time frames of competition assays, inhibition of S1P production with the sphingosine kinase inhibitor SKI II was toxic to both the losers and the winners (Fig.2.2c). While secreted S1P can signal in an autocrine and paracrine manner via its receptors, intracellular S1P acts as an anti-apoptotic agent (Maceyka *et al*. 2012). It is likely the long term disruption of this intrinsic pathway that triggered the death of the MDCK cells.

2.4. **Discussion**

2.4.1. Mechanical cell competition

Earlier experiments by Laura Wagstaff indicated that, during competition, *scribble*-deficient cells are compressed by normal cells. They also suggested that the mutant cells may be hypersensitive to density and consequently die when crowded. In this chapter, I directly proved that enforced compaction is sufficient to kill *scribble*-deficient cells. We termed the phenomenon in which loser cells are killed by mechanical means "mechanical cell competition".

A key function of epithelial is to provide a physical barrier between two different environments. It is therefore of particular importance that individual epithelial cells are able to withstand the mechanical stress to which they are subjected. Mechanical competition could provide a means by which cells with sub-optimal mechanical properties are actively eliminated by their normal neighbours, even before they can endanger

tissue integrity. Indeed, in both competition models described in this chapter, loser cells had an altered shape and thereby mechanical properties.

Levayer *et al*. (2016) recently described that Ras-mutant clones in *Drosophila* notum are able to compress and kill the surrounding normal cells. However, unlike in our model, they did not suggest that the normal cells had to undergo a morphological change in order to become losers. This might suggest the existence of an alternative mechanism of mechanical competition, in which super-competitors acquire a "mechanical advantage", and hence become more resistant to mechanical stress than normal cells. Alternatively, transformed cells might, by short-range signalling, change the mechanical properties of the neighbouring normal cells, rendering them more susceptible to mechanical out-competition.

2.4.2. Death and extrusion of the loser cells

Compression experiments conducted in absence of a winner cell population revealed that overcrowding is sufficient to trigger apoptosis of *scrib*$^{KD+TET}$ cells. This agrees with previous observations that apoptosis is the major means by which these loser cells are eliminated (Norman *et al*. 2012). Inhibition of S1P2 and Piezo1 signalling did not affect the elimination of *scrib*$^{KD+TET}$ cells, further supporting earlier observations that extrusion, although present during *scribble* competition, is not required for the elimination of loser cells. Nevertheless, extrusion pathways may play another role in mechanical competition. Laura Wagstaff observed that *scrib*$^{KD+TET}$ cells are actively compacted by surrounding normal cells (Fig.1.4). It is conceivable that the mechanisms which squeeze e.g. Ras- and Src-transformed MDCK cells out of an epithelium, are the same as those that compact *scribble*-deficient cells, but that *scrib*$^{KD+TET}$ cells, due to their hypersensitivity to crowding, die before they had a chance to be extruded. This hypothesis was not supported by the obtained results. If the extrusion pathways facilitated compaction of losers, then cell density in loser clones should not have exceeded the density of winners when these pathways were

blocked. This was not the case in cultures treated with S1P2 or Piezo1 inhibitors, suggesting that neither molecule is involved in any stage of competition. Nevertheless, it might be interesting to test whether molecules required for extrusion of transformed cells, e.g. filamin A, Rho and Rho kinase (Kajita *et al*. 2014), have conserved functions in competition.

Chapter 3. TRANSCRIPTIONAL PROFILING REVEALS ELEVATED P53 SIGNALLING AS THE KEY SIGNATURE OF *SCRIBBLE*-DEFICINET LOSER CELLS

3.1. Introduction

Despite the recent explosion of studies on cell competition, still relatively little is known about what defines cells as losers. Having recently proven that *scribble*-deficient cells are hypersensitive to compaction, and that this property is responsible for their mechanical out-competition, I set out to identify the molecular signature of prospective loser cells in order to establish which pathways were responsible for their mechanical loser status.

To address this question I decided to focus on "naïve" loser cells, i.e. on cells from homotypic cultures that never came in contact with a winner cell population. In this way, I attempted to identify genes and pathways which are altered cell-autonomously, and which trigger cell competition. If I had instead isolated the losers from a co-culture with normal cells, it would have been difficult to distinguish between those genes that trigger competition, and those involved in later, effector phases of competition.

The signature of the loser cells may encompass alterations in transcriptional programs, protein levels, posttranslational modifications, protein localisation and changes in non-protein cellular components. I decided to concentrate on characterising the transcriptomes of loser cells, both for technical convenience, and because Microarray analysis has successfully identified *Drosophila* Flower as a determinant of loser or winner cell status (Rhiner *et al*. 2010). Although other RNA species have been implicated in cell competition (Eichenlaub *et al*. 2016), here I concentrated on sequencing the mRNA pool.

I used RNAseq to compare the transcriptomes of control and *scribble*-deficient MDCK cells. The used cell line carries an anti-*scribble* shRNA sequence (*scrib*^{KD}), inducibly expressed in presence of tetracycline (*scrib*$^{KD+TET}$). In absence of tetracycline these cells behave as wild-types (*scrib*$^{KD-TET}$). By comparing transcriptomes of *scrib*$^{KD+TET}$ and *scrib*$^{KD-TET}$ cells, I characterised the effects of *scribble* RNAi on transcription, while reducing the contribution of genomic variability to the obtained results. Furthermore, I also analysed the transcriptome of a sub-clone of *scrib*$^{KD+TET}$ cells that do not behave as losers (*scrib*^{RES}, which had been previously isolated in the lab). By comparing the transcriptomes of *scrib*$^{KD+TET}$ and *scrib*$^{RES+TET}$ cells, I attempted to enrich for those genes affected by *scribble* silencing that correlate with the loser status.

3.2. **Optimising experimental conditions**

To ensure that the cells were harvested for sequencing at an optimal time, I first established the timing of competition in co-culture experiments (Fig.3.1). For competition to start, sufficient time has to pass since tetracycline has been added, but the timing also depends on when individual clones begin to touch, and hence on the initial plating density. Therefore, I used the same total plating densities and added tetracycline at the same time for both RNAseq and competition assays. I used a 1:9 ratio of mutant to wild-type cells. The number of *scrib*$^{KD+TET}$ cells increased stably until approximately 55 hours after plating. Afterwards, the number of mutant cells progressively declined (Fig.3.1a), suggesting ongoing competition. Indeed, the first *scrib*$^{KD+TET}$ clones began to die shortly after 55 hours after plating, with the onset of death in the majority of clones at 70-80 hours after plating (Fig.3.1b). Based on these results, and to assure that all cells had fully acquired the changes caused by *scribble* silencing, but to limit the

effects of potential competition between *scrib*$^{KD+TET}$ cell sub-clones, I decided to harvest all samples at precisely 67 hours after plating.

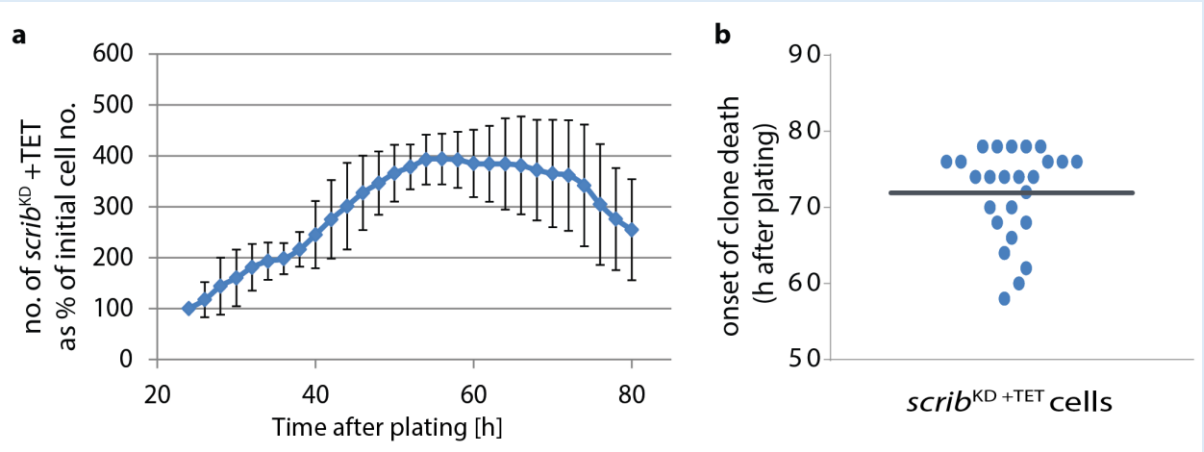


Figure 3.1. Establishing the timing of *scribble* competition.
(**a-b**) GFP-labelled *scrib*^{KD} cells and unlabelled wild-type cells were plated at a 1:9 ratio and at a total of 1.3 Million cells per 10 cm plate in medium supplemented with tetracycline. (**a**) Quantification of growth rate of *scrib*^{KD} cells from time-lapse movies, showing average (±SD) number of *scrib*^{KD} cells per clone. (**b**) Quantification showing the onset of death in *scrib*^{KD} clones. Each dot represents a single clone. Black bar = mean.

3.3. **Quality control**

I performed three independent RNAseq experiments, each with two biological replicates per condition. To confirm the effectiveness of silencing, each experiment was accompanied by corresponding competition assays. As additional control, I used the RNAseq results to confirm the silencing of *scribble*. In each sample, I observed an over two fold reduction in the expression of *scribble* in presence of tetracycline (28±3.68 RPKM for *scrib*$^{KD-TET}$ *vs* 12.91±2.69 RPMK for *scrib*$^{KD-TET}$; RPKM = Reads Per Kilobase of transcript per Million mapped reads). As predicted by Western blot analysis carried by others, there was no significant difference in the expression of *scribble* between *scrib*$^{KD+TET}$ cells and *scrib*$^{RES+TET}$ cells (13.31±1.23 RPMK for *scrib*$^{RES+TET}$).

All cell culture and cDNA libraries were prepared by myself and submitted for next-generation sequencing. Sequencing results were processed and analysed by, or with the help of, Charles Bradshaw and George Allen from the Gurdon Institute Core Bioinformatics team. To verity the reproducibility of the obtained data, we performed cluster dendrogram analysis. As expected, biological replicates from a single experiment always clustered together, confirming reproducibility within experiments (Fig.3.2). Unexpectedly, gene expression varied more from experiment to experiment, than between different experimental conditions (Fig.3.2). There are several likely explanations: (1) The effect of genomic variability between cells used in different experiments outweighed the effect of treatments. In each experiment I used a different sub-clone of *scrib*^{KD} cells. Nevertheless, this was not likely a major factor, as all *scrib*^{RES} cells were derived from the same sub-clone, yet they did not cluster together, nor did all of them cluster with the parental *scrib*^{KD} sub-clone (Fig.3.2, *scrib*^{RES} cells were derived from the sub-clone used in experiment-3, depicted in red). (2) Unequal levels of silencing of *scribble*. This was not the case, as *scribble* was silenced to a similar degree in all samples (average 28±3.68 RPKM and

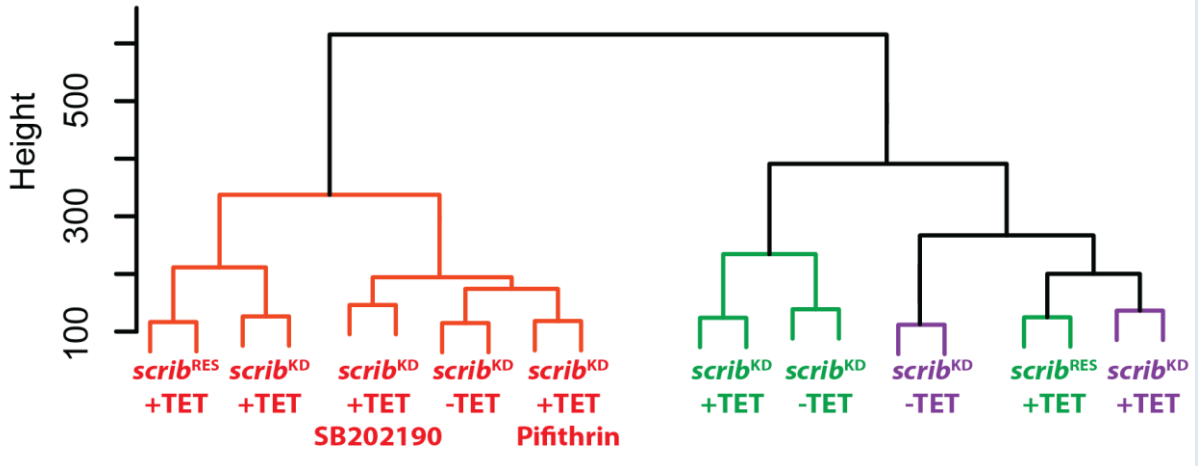


Figure 3.2. Cluster dendrogram of all RNAseq samples.
Colours represent independent experiments: experiment-1 (purple), experiment-2 (green) and experiment-3 (red). Lowest branches correspond to biological duplicates of treatments from a single experiment.

12.91±2.69 RPMK for *scrib*$^{KD-TET}$ and *scrib*$^{KD+TET}$ respectively). (3) Variability

in other tissue culture conditions, such as temperature or age of the cultures. (4) Differences in preparation or processing of the cDNA libraries and sequenced data. To address the variability between results obtained in different experiments, we applied the following strategy to compare transcriptomes of *scrib*$^{KD-TET}$, *scrib*$^{KD+TET}$ and *scrib*^{RES} cells. We first generated lists of differentially expressed transcripts for individual experiments, and then compared those lists to identify transcripts differentially expressed in all relevant experiments.

3.4. Obtaining the molecular signature of *scribble*-deficient prospective loser cells

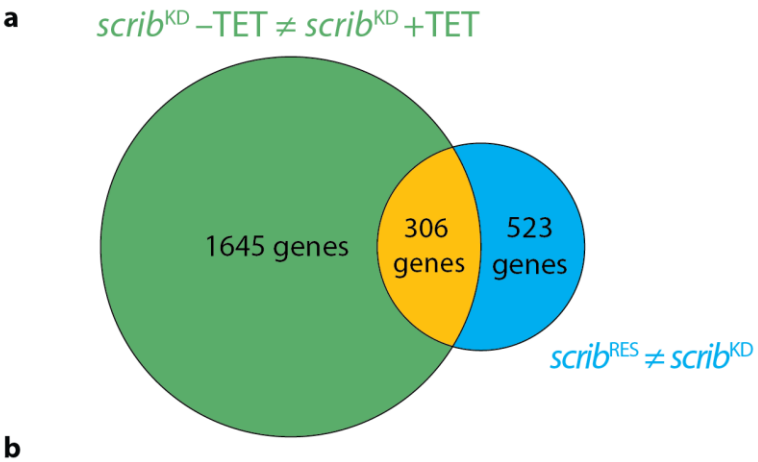
To generate lists of differentially expressed genes, comparisons were made between pairs of conditions, each with at least four replicates. For a transcript to be included, counts per million had to be above 10 for all samples in at least one condition and within 2-fold between replicates. The lists included genes with logarithmic fold change (log_2FC) of over 0.5. We identified 1645 genes affected by silencing of *scribble* (*scrib*$^{KD+TET}$ *vs scrib*$^{KD-TET}$ cells; Fig.3.3a, green circle and Supplementary Data-1). The *scrib*$^{KD+TET}$ transcriptome was substantially closer to *scrib*$^{RES+TET}$ (with 523 differentially expressed genes; Fig.3.3a, blue circle and Supplementary Data-2). An intersection of those two lists (Fig.3.3a, orange section, and Supplementary Data-3) includes 306 genes that were differentially expressed specifically in those *scrib*$^{KD+TET}$ cells that are susceptible to out-competition.

I then carried out Gene Ontology enrichment analysis on the list of 306 genes, using David Bioinformatics Resources (Huang *et al*. 2009), in particular KEGG pathway analysis (Kanehisa & Goto 2000; Kanehisa *et al*. 2012). Gene Ontology term enrichment analysis highlighted p53 signalling as the top functionally enriched pathway (Fig.3.3b). Among other enriched terms (Fig.3.3b) were adherens junctions. The role of the main component of epithelial adherens junctions, E-cadherin, in cell competition, has been

investigated by others from our group (Wagstaff *et al*. 2016; Fig.1.4). Junction-associated proteins and other cell-membrane proteins are of particular interest, as their location suggests potential involvement in cell-cell interactions. Interestingly, several enriched Gene Ontology terms included surface receptors such as TGFβ receptor-2 (two-fold enrichment in *scrib*$^{KD+TET}$ *vs* *scrib*$^{KD-TET}$ cells, completely rescued in *scrib*$^{RES+TET}$; Supplement-3) and FGF receptor-2 (over two-fold reduction in *scrib*$^{KD+TET}$ *vs* *scrib*$^{KD-TET}$ cells, rescued in *scrib*$^{RES+TET}$; Supplementary Data-3), together with its ligand FGF-21. Among pathways regulated by both TGFβ and FGF signalling, is the MAPK pathway, including p38 MAPK (Koul *et al*. 2013), which has been previously implicated in *scribble* competition (Norman *et al*. 2012).

In addition to generating lists of differentially expressed genes, here and throughout my PhD I used the RNAseq data to investigate the expression of selected candidate genes, to complement other experimental approaches. Among those genes where several proteins known to trigger competition in other systems. Myc, whose involvement has been reported in many types of competition, but not in *scribble* competition, has was expressed inconsistently between samples, with no apparent change in expression pattern. Similarly, the expression of ribosomal proteins did not differ between *scrib*$^{KD+TET}$, *scrib*$^{KD-TET}$ and *scrib*$^{RES+TET}$ cells (Fig.3.3c). Chen and colleagues (2012) reported the involvement of the Hippo pathway in *Drosophila scribble* competition. Hippo pathway was not enriched in Gene Ontology analysis, however this does not preclude posttranscriptional alterations, or involvement at later stager of competition.

a



b

KEGG pathway term	PValue	Genes
p53 signalling pathway	0.0017	P21↑, SERPINB5↑, SERPINE1↑, MDM2↑, CCNG1↑
Pathways in cancer	0.0073	E2F1↓, FGFR2↓, P21↑, PTGS2↑, PAX8↓, CREBBP↓, TGFBR2↑, NKX3-1↓, MDM2↑, FGF21↑, ABL1↓
Adherens junction	0.0155	SORBS1↑, PVRL3↓, CREBBP↓, TGFBR2↑, LMO7↑
MAPK signalling pathway	0.0504	FGFR2↓, TGFBR2↑, NR4A1↓, HSPB1↓, NFATC4↓, FGF21↑, PPP3CA↑, SRF↑
VEGF signalling pathway	0.0719	PTGS2↓, HSPB1↓, NFATC4,↓ PPP3CA↑
Cell cycle	0.0793	E2F1↓, P21↑, CREBBP↓, MDM2↑, ABL1↓

c

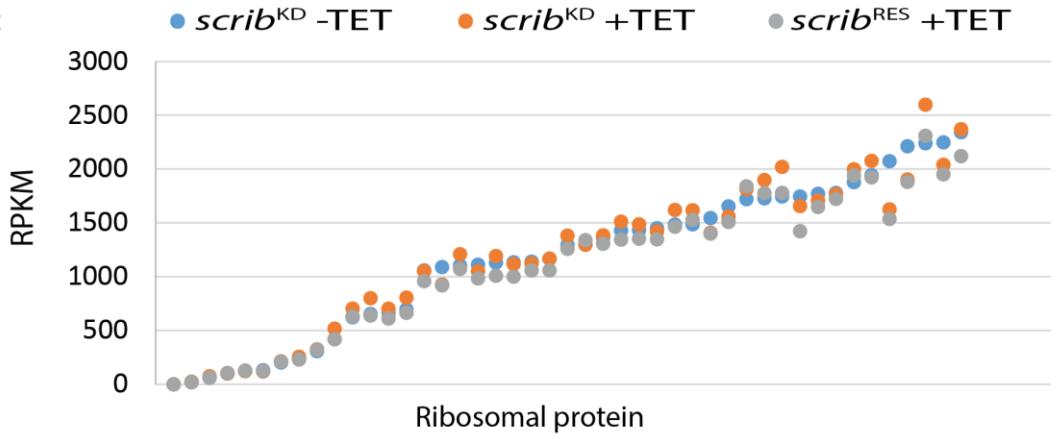


Figure 3.3. Molecular signature of *scribble*-deficient loser cells.
(**a**) Transcriptional profiling of *scrib*^{KD} cells without TET *versus* *scrib*^{KD} cells with TET (green), and of *scrib*^{KD} cells with TET versus *scrib*^{KD} cells resistant to competition (*scrib*^{RES}) with TET (blue). Orange intersection represents genes that are differentially expressed +/- TET and between competition-sensitive and competition-resistant *scribble*-deficient cells. (**b**) List of pathways functionally enriched in the orange intersection. Arrows represent increase (green) or reduction (red) in expression in *scrib*$^{KD+TET}$ *versus* *scrib*$^{KD-TET}$ cells. (**c**) Expression of genes encoding ribosomal proteins is not affected by silencing of *scribble*. Each dot represents the expression of a single transcript.

3.5. **RNAseq revealed elevation of p53 signalling in *scribble*-deficient loser cells**

Gene Ontology term enrichment analysis highlighted p53 signalling as the top functionally enriched pathway (Fig.3.3b). Although the expression of p53 itself was not altered by silencing of *scribble*, a number of known transcriptional targets of p53 were upregulated in *scribble*-deficient cells, suggesting activation of p53 (Fig.3.3b, Table.3.1 and Supplementary Data-3). Strikingly, for most p53 targets, expression of p53 in *scrib*$^{RES+TET}$ cells reverted back to levels comparable to those observed in *scrib*$^{KD-TET}$ (Table.3.1 and Supplementary Data-3), indicating that upregulation of p53

Table 3.1. p53 targets are upregulated selectively in competition-sensitive *scribble*-deficient cells.

Presents fold change (FC) in expression between cell treated with TET and untreated *scrib*^{KD} cells, for selected p53 targets. Treated (+TET) samples include: *scrib*^{KD} cells, *scrib*^{KD} cells resistant to competition (*scrib*^{RES}), and *scrib*^{KD} cells cultured with either a p53 inhibitor (Pifithrin- α) or a p38 inhibitor (SB202190).

Official gene indicator	Other gene names	FC *scrib*$^{KD+TET}$	FC *scrib*$^{RES+TET}$	FC *scrib*$^{KD+TET}$ +Pifithrin	FC *scrib*$^{KD+TET}$ +SB202190
Cdkn1a	cyclin-dependent kinase inhibitor 1A (p21)	3.57	1.48	3.58	2.69
Ccng1	cyclin G1	2.78	1.53	3.08	3.34
Mdm2	MDM2 oncogene, E3 ubiquitin protein ligase	2.08	1.14	2.11	2.12
Serpinb5	serpin peptidase inhibitor, clade B (ovalbumin), member 5	1.92	1.08	2.22	2.55
Serpine1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	1.89	2.89	0.77	1.13

is specifically present in those *scribble* RNAi cells that will behave as losers in mixed cultures.

In addition to analysing the transcriptomes of *scrib*$^{KD+TET}$, *scrib*$^{KD-TET}$ and *scrib*$^{RES+TET}$ cells, I conducted a single experiments, I which I sequenced transcriptomes of *scrib*$^{KD+TET}$ cells cultured in presence of the p53 inhibitor Pifithrin-α, or the p38 inhibitor SB202190 (Table.3.1). Pifithrin-α has been shown in our lab to block *scribble* competition. Surprisingly though, it did not alter the expression of most p53 targets, compared to untreated *scrib*$^{KD+TET}$ control (Table.3.1). Similarly, the p38 inhibitor also did not affect the expression of most p53 targets (Table.3.1). Since p38 signalling is known to be involved in competition (Norman *et al*., 2012), this suggests that: (1) p38 affects the loser cell status independently of p53, or (2) that it only affects p53 signalling in the effector phase of competition or (3) that it affects the non-transcriptional function of p53.

3.6. **Discussion**

3.6.1. Molecular signature of loser cells

I employed RNAseq to characterise the molecular signature of prospective loser cells prior to competition. To the best of my knowledge, this is the first comprehensive attempt to unravel the molecular mechanism of cells competition in an adult mammalian tissue. By comparing transcriptomes of normal (*scrib*$^{KD-TET}$), *scribble*-deficient (*scrib*$^{KD+TET}$), and *scribble*-deficient MDCK cells resistant to competition (*scrib*$^{RES+TET}$), I obtained a list of 306 candidate genes with a possible role in triggering cell competition. I then employed Gene Ontology analysis to identify those pathways and processes that are likely miss-regulated in loser cells prior to competition. While I and others in our group concentrated on further characterising the role of p53 in competition, other functionally enriched pathways bare further studies. Moreover, in addition to a candidate-based approach, a genetic screen could

be developed to address the role of the 306 genes in cell competition. As a caveat, this list includes both genes in which the effects of tetracycline are reversed in competition-resistant cells (i.e. expression levels are similar between *scrib*$^{KD\text{-}TET}$ and *scrib*$^{RES+TET}$ cells), and those in which the effects of tetracycline treatment are exacerbated in *scrib*^{RES} cells. Only the first group is likely to include genes involved in competition. Conversely, although useful as a starting point for future analysis, the list of 306 genes is not expected to include all transcripts that contribute to the loser signature. For instance if several pathways signal in parallel to regulate a downstream effector, it is more likely that resistance to competition will be gained by altering transcription of this downstream effector, rather than by simultaneous changes in transcription in all of the redundant pathways. For this reason, only the downstream effector is likely to be found among the 306 genes.

3.6.2. p53 in *scribble* cell competition

Gene Ontology analysis identified p53 signalling as the most enriched pathway linked to competition. Strikingly, a number of targets of p53 were upregulated in competition-sensitive, but not in competition-resistant *scribble*-deficient cells, suggesting that p53 was up-regulated specifically in prospective loser cells, and that this up-regulation was required to trigger *scribble* competition. This hypothesis was later confirmed by myself and others from our group, as describe in the following chapters. Having successfully identified p53 as a key player in *scribble* cell competition validates the experimental approach and promises further discoveries, were the remaining candidate genes to be characterised.

The p53-inhibitor Pifithrin-α blocked out-competition of *scribble*-deficient cells, but did not affect the lever of expression of known targets of p53 in *scribble*-deficient cells naïve to competition. Possible explanations of this apparent contradiction include: (1) Pifithrin-α does not prevent p53 from controlling transcription, but inhibits transcription-independent functions of

p53. (2) Pifithrin-α affected transcription of only a subset of p53 targets, and these targets are key for loser cell signature. This seems unlikely, since the only identified target of p53 whose up-regulation was affected by Pifithrin-α (Serpine1), was not rescued in *scrib*$^{RES+TET}$ cells, and hence is unlikely to be involved in competition(Table-3.1). (3) The effect of Pifithrin-α on competition is unrelated to p53 signalling. (4) Pifithrin-α does not alter the loser signature, but affects later stages of competition. Pifithrin-α has been reported to inhibit expression from a p53-regulated promoter (Komarova and Gudkov, 2000), and abolished activation of the p53-target gene, p21 (Zhang *et al*. 2015). Nevertheless, the relative importance of transcription-related and -unrelated p53 signalling in early and late stages of competition bares further examination.

Chapter 4. COMPACTION TRIGGERS APOPTOSIS VIA P53

4.1. p53 signalling is required for elimination of *scribble*-deficient cells by mechanical compression

Following on my transcriptional data, Laura Wagstaff confirmed a cell-autonomous elevation of p53 in *scrib*$^{KD+TET}$ cells by immunoflurescent staining and by Western blot analysis. Importantly, she then demonstrated that p53 was further boosted in *scrib*$^{KD+TET}$ cells surrounded by normal cells. This observation led us to hypothesize that p53 may play a dual role in mechanical competition, first by identifying cells as losers, and later by triggering apoptosis in response to compaction. I therefore went on to test both of these roles directly. To directly prove that p53 is required to induce apoptosis in response to overcrowding, I analysed the response of *scrib*$^{KD+TET}$ *p53*$^{-/-}$ cells to compression in absence of wild-type cells. When compressed on a PDMS membrane, the double mutants commonly reached densities in excess of 150 cells/field, suggesting a reduced sensitivity to crowding, when compared to *scrib*$^{KD+TET}$ cells. Moreover, while the frequency of cell death in sub-confluent cultures (compaction-independent death) did not significantly differ between *scrib*$^{KD+TET}$ and *scrib*$^{KD+TET}$ *p53*$^{-/-}$ cells (1.22±1.69% apoptotic events/imaged field for single mutants and 1.09±2.24% for double mutants; Fig.4.1), in compressed high density cultures three times fewer *scrib*$^{KD+TET}$ *p53*$^{-/-}$ stained positive for activated Caspase-3 than *scrib*$^{KD+TET}$ cells (respectively 2.84±1.24% and 8.43±2.98% apoptotic events per imaged field; Fig.4.1). Together, the comparable background levels of apoptosis and the strong reduction in death upon compression suggest that p53 is required specifically for compaction-induced death of *scrib*$^{KD+TET}$ cells. Interestingly, the rescue from death upon compression was not complete (1.09±2.24% in low density *versus* 2.84±1.24% in high density *scrib*$^{KD+TET}$ *p53*$^{-/-}$ cultures; Fig.4.1),

suggesting either residual p53 activity or that a pathway parallel to p53 signalling contributes to the response of mechanical losers to overcrowding.

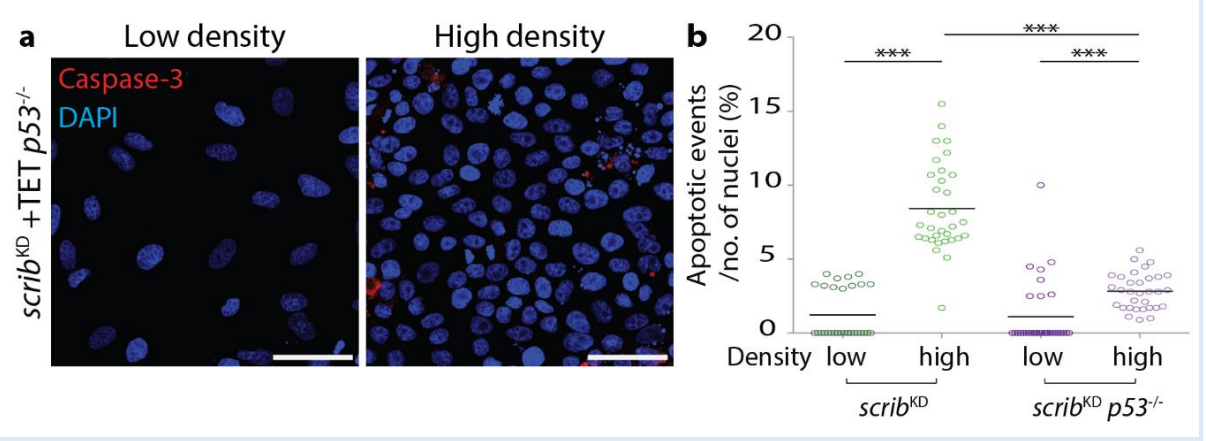


Figure 4.1. Compaction kills *scribble*-deficient cells by activating p53 signalling. *scrib*$^{KD+TET}$ cells or *scrib*$^{KD+TET}$ *p53*$^{-/-}$ cells were cultured at two densities (confluence and sub-confluence) on a stretched PDMS membrane. Release of the stretch compressed only the high density cultures. The samples were fixed five hours after the release of stretch. Cell death was detected by immunofluorescent staining against activated Caspase-3. (**a**) Representative confocal images of *scrib*$^{KD+TET}$ *p53*$^{-/-}$ low density (left panel) and high density (right panel) cultures. (**b**) Quantification of cell death (activated Caspase-3) for *scrib*$^{KD+TET}$ vs *scrib*$^{KD+TET}$ *p53*$^{-/-}$ cells +/- compression; black bars = mean. *** p<0.0005 by KS test.

4.2. **Activation of p53 turns wild-type cells into mechanical losers**

Having established that p53 signalling is required for the death of *scrib*$^{KD+TET}$ upon compaction I then asked whether sub-lethal elevation of p53 signalling is sufficient to induce hypersensitivity to crowding to otherwise wild-type cells. This was directly based on some experiments from Laura Wagstaff, who showed that mixed wild-type and p53$^{-/-}$ MDCK cells could compete. Specifically, she showed that in the absence of an external source of stress, p53 signalling levels were low and there was no competition in the mixed cultures. However, with sub-lethal concentrations of Nutlin-3, which prevents the MDM2-p53 interaction leading to p53 activation, wild-type cells

were killed in the mixed culture. I then asked whether this out-competition occurs because of hypersensitivity to crowding.

Promisingly, much like silencing of *scribble*, treatment with low doses of Nutlin-3 caused flattening of MDCK cells in monoculture (Fig.4.2a, left panel) and compaction and elimination in co-culture with winner cells (Wagstaff *et al*. 2016).To directly test the sensitivity of Nulin-3-treated cells to mechanical compression, I again employed the stretch-release method, plating normal cells at high and at low density in presence or absence of Nutlin-3 and staining for activated-Caspase-3-positive cells. Similarly to silencing of *scribble*, mild activation of p53 induced low levels of death even without compaction (low density cultures; 0.39±0.69% apoptotic events/imaged field for untreated *vs* 1.66±2.61% for Nutlin-3-treated cells; Fig.4.2a-b). As expected, untreated cells were resistant to compaction, with no significant difference in the frequency of apoptotic cells between low and high density cultures (0.39±0.69% apoptotic events/imaged field at low density and 0.52±0.50% at high density; Fig.4.2a-b). On the contrary, cells treated with Nutlin-3 died 3.6 times more frequently when compressed, than in un-compressed control (1.66±2.61% apoptotic events/imaged field at low density *vs* 5.90±2.56% at high density; Fig.4.2a-b), suggesting that elevation of p53 increases sensitivity to density and is sufficient to trigger apoptosis upon compaction.

4.3. **p53-high 16Hbe cells, but not Eph4 cells, are hypersensitive to compaction**

I further asked whether the p53-driven hypersensitivity to compaction is conserved between different epithelial cell lines. Indeed, cells from an immortalised bronchial epithelial cell line, 16Hbe were more sensitive to compaction when treated with sub-lethal doses of Nutlin-3, than when treated with DMSO control (Fig.4.2c-e). Unlike in MDCK cells, Nutlin-3 had no effect on cell viability in uncompressed cultures (17423±29872

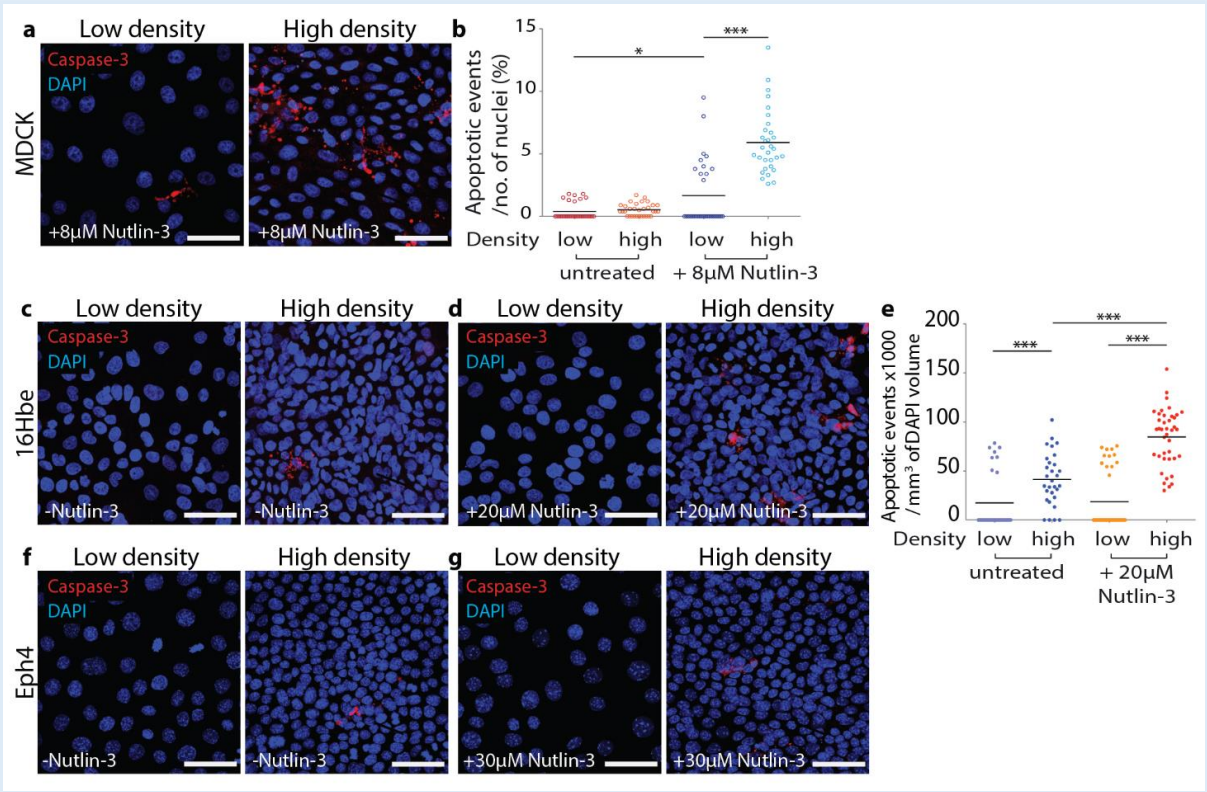


Figure 4.2. Compaction kills cells with elevated p53 signalling.
Wild-type cells were cultured at two densities (confluence and sub-confluence) +/- a p53 inducer (Nutlin-3) on a stretched PDMS membrane. Release of the stretch compressed only the high-density cultures. The samples were fixed five hours after the release of stretch. Cell death was detected by immunofluorescent staining against activated Caspase-3. (**a**) Representative confocal images of MDCK cells + Nutlin-3 in low density (left panel) and high-density (right panel) culture. (**b**) Quantification of cell death (activated Caspase-3) for MDCK cells +/- compression and +/- Nutlin-3. **(c-e)** Representative confocal images of 16Hbe cells +/- compression + DMSO (control) (**c**) or + Nutlin-3 (**d**) with corresponding quantification (**e**). (**f, g**) Representative confocal images of Eph4 cells +/- compression + DMSO (control) (**f**) or + Nutlin-3 (**g**). Black bars in graphs = mean. * $p<0.05$, *** $p<0.0005$ by KS test. Scale bars = 50 μm.

apoptotic events/mm^3 of DAPI volume for DMSO-treated low density cultures and 18600±29682 for Nutlin-3-treated low density cultures; Fig.4.2c-e). In compressed cultures, the frequency of apoptotic cells doubled in Nutlin-3-treated samples (41467±26750 apoptotic events/mm^3 of DAPI volume for DMSO-treated high density cultures and 84658±29105 for Nutlin-3-treated high density cultures; Fig.4.2c-e), suggesting that activation of p53 signalling is sufficient to render 16Hbe cells hypersensitive to compaction. Notably, unlike wild-type MDCK cells, wild-type 16Hbe cells

underwent apoptosis upon compaction even in absence of an external activator of p53 (17423±29872 apoptotic events/mm^3 of DAPI volume in low density cultures and 41467±26750 in high density DMSO-treated cultures; Fig.4.2c,e). It is possible that compaction alone is sufficient to elevate p53 signalling in 16Hbe cells to lethal levels. Alternatively, an additional, parallel mechanism of elimination of supernumerary cells may exist.

The hypersensitivity to compaction of p53-high 16Hbe cells demonstrates that this effect is not unique to MDCK cells. However, it is not conserved in all immortalised epithelial cell lines with functional p53 signalling. Eph4 mouse breast epithelial cells did not die at low density (uncompressed cultures) either in presence or in absence of Nutlin-3 (Fig.4.2f). Enforced compaction sporadically triggered death, but with no appreciable difference between Nutlin-3-treated and -untreated samples (Fig.4.2g). This suggests that elevation of p53 had no effect, or had an undetectably mild effect on the sensitivity of Eph4 cells to compaction.

4.4. **Discussion**

4.4.1. p53 in mechanical competition

The involvement of p53 in mechanical cell competition reveals a new role of p53 signalling in the elimination of stressed cells.

We demonstrated that p53 signalling is required for the elimination of *scrib*$^{KD+TET}$ cells by mechanical means. By employing an enforced-compression assay I demonstrated that p53 affects the sensitivity to crowding, thereby rendering p53-high cells more sensitive to death by compaction. We further demonstrated that elevation of p53 is sufficient to alter shape of normal MDCK cells, rendering them hypersensitive to density and to elimination by mechanical means. While elevation of p53 had been previously reported as a determinant of loser cell status in the bone marrow

(Bondar & Medzhitov 2010; Marusyk *et al*. 2010), the molecular mechanism of this process have not been investigated. It would be therefore interesting to test whether all p53 competition occurs by mechanical means.

Since p53 is a universal stress sensor, we asked whether mechanical p53 competition is a common mechanism for the elimination of stressed epithelial cells. For this reason I tested the effect of p53 signalling on sensitivity to compression in several cell lines. I selected immortalised cell lines with functional p53 signalling and epithelial morphology, and was able to reproduce the hypersensitivity to enforced compaction in the 16Hbe bronchial epithelial cells (see also Chapter 6. on the establishment of a p53-induced competition assay from primary bronchial epithelial cells). This indicates that p53 competition may be indeed conserved between different epithelial cell lines and between different tissues. However I was unable to observe the same effect in the Eph4 cells. p53 competition is therefore either not conserved in all tissues, or was lost as a result of the immortalization process.

4.4.2. p53 in cancer

In addition to its significance as a homeostatic process, the discovery of p53 competition may have major consequences for cancer biology, as p53 is the single most commonly mutated protein in cancer. p53 competition might be a means by which tumours obtain space to expand, or invading cells secure a foothold to colonise a tissue. Our studies indicate that p53-deficient cells can efficiently out-compete normal cells, but that this only happens when p53 signalling is elevated in the normal cells. It would be therefore interesting to test whether the microenvironment of a growing tumour is capable of elevating p53 in the surrounding tissues and of rendering them into mechanical losers. Alternatively, p53 competition could promote tumour expansion in tissues subjected to external stressors such as cigarette smoke in the lungs or UV irradiation of the skin.

Mechanical competition does not account for all functions of p53 signalling in tumorigenesis, as p53-deficient mice develop cancer with a much higher frequency than normal animals even in absence of mosaicism (Christophorou *et al*. 2005), but it may be a major contributor to cancer development.

Chapter 5. PRIMARY CULTURES OF MOUSE TRACHEAL EPITHELIAL CELLS PROVIDE A NEW MODEL TO STUDY P53-DRIVEN MECHANICAL CELL COMPETITION

5.1. Introduction

The discovery of p53-driven mechanical competition in MDCK cell culture may lead to valuable insights into cancer biology. However, before any conclusions can be drown, it is necessary to prove that mechanical competition is not artefactual and/or MDCK cell-line specific. In Chapter 4, I described that mild elevation of p53 signalling is sufficient to induce hypersensitivity to compaction of immortalised human bronchial epithelial 16Hbe cells (Fig.4.2c-e). This suggests that differential activity in p53 signalling might be sufficient to trigger competition among tracheal epithelial cells. I decided to further investigate this possibility using a more *in vivo*-like primary tracheal cell culture system.

The airways are lined with pseudo-stratified mucociliary columnar epithelia, which can be modelled *in vitro*. I chose to study competition in a differentiated mouse bronchial epithelial culture (Fig.5.1; You and Richer, 2002) routinely used by my co-supervisor, Emma Rawlins. There are many reasons for choosing this system: (1) The likelihood of p53-driven mechanical competition based on earlier experiments on 16Hbe cells. (2) The technical expertise provided by the Rawlins group. (3) The well-established role of p53 in lung cancer. (4) Differentiated tracheal epithelial cultures are stable for many months and hence suitable for long experiments, which were required to study competition in other mammalian adult tissues (Oertel *et al*. 2006; Bondar & Medzhitov 2010; Villa Del Campo *et al*. 2014). (5) The epithelium is mono-layered and hence suitable for live

imaging. (5) The easy access to the tracheal epithelium *in vivo* for purposes such as treatment, will help to validate *in vitro* results. (6) The composition of mouse tracheal epithelium is relatively simple, with only three dominant cell types (Fig.5.1a). Yet, it is more complex than most tissues that had been used to study competition in *Drosophila* and mammals, and may give valuable insights into the respective roles of stem and differentiated cells in cell competition.

5.2. **Establishing primary mouse tracheal epithelial cell cultures**

Before proceeding to model competition in mouse tracheal epithelial cell (MTEC) culture, I decided to verify the quality of my cultures. *In vivo*, the mouse tracheal epithelium consists mainly of Basal cells, Secretory cells, and Ciliated cells, and this composition can be reproduced *in vitro* (You and Richer, 2002). Shortly after plating on a porous membrane submerged in a nutrient reach medium, the cells rapidly spread and proliferate (You and Richer, 2002), to form a confluent monolayer (Fig.5.1a). In time, the density of the culture increases until it reaches a homeostatic level. At this stage, differentiated cells appear, and beating cilia can be observed under a light microscope (Fig.5.1a). A mature culture can be then maintained nearly indefinitely. I judged a culture ready for experiments when cell density stabilised and when ciliated cells appeared.

Mouse Basal cells are the stem cells of mouse tracheal epithelium. They generate differentiated cells during postnatal growth, and in adult trachea both during steady state and following injury (Rock *et al*. 2009). *In vivo*, Basal cells can be found in the mouse trachea and throughout human airways, and can be detected by the expression of Trp-63 (p63) and keratins 5 (KRT-5) and 14 (KRT-14). As expected, I was able to easily detect Basal cells in young, rapidly proliferating epithelia that did not yet

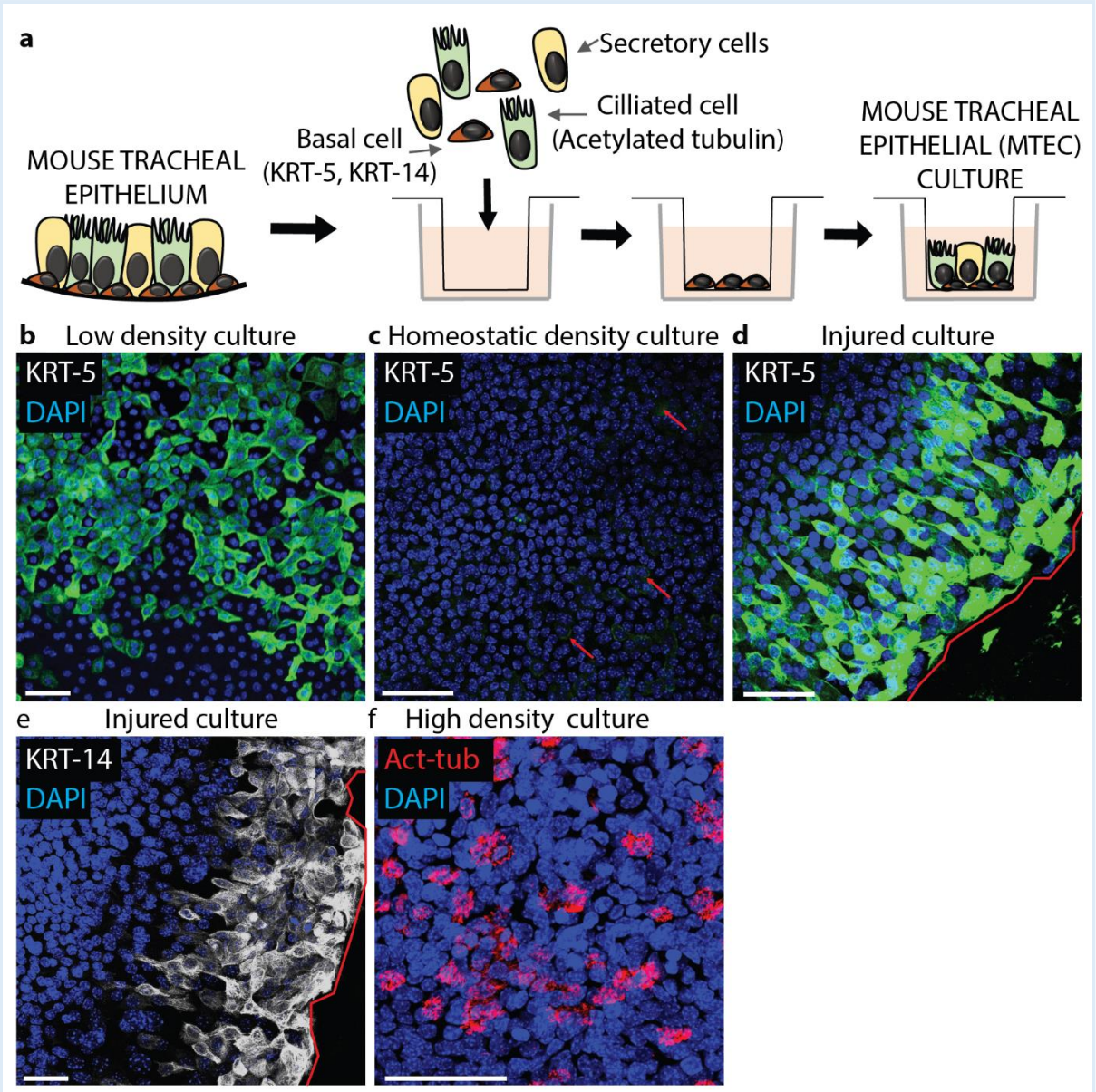


Figure 5.1. Primary mouse tracheal epithelial cell culture (MTEC).
(**a**) Establishing an MTEC culture. Mouse tracheal epithelium is composed of Basal (stem/progenitor) cells (brown), Secretory cells (yellow) and Ciliated cells (green). Early, sub-confluent cultures are formed by Basal cells. Confluent, mature cultures contain both Basal and differentiated cells. (**b-d**) Basal cell marker Keratin-5 (KRT-5) is abundantly expressed in young cultures at below-homeostatic density (**b**), but detected only at low levels and in a subset of cells in mature cultures (**c**; red arrows). (**d**) Scratching (right from red line) culture as in **c** restored high levels of KRT-5 in the vicinity of the wound. (**e**) Expression of Basal cell marker Keratin-14 was also low in mature cultures, but boosted near wound edge (red line). (**f**) Staining against Acetylated Tubulin-α (Act-tub) detected ciliated cells in mature epithelia. Scale bars = 50 μm.

reach full homeostatic density (KRT-5 staining, Fig.5.1b). In mature epithelia, Basal cells are expected to make up approximately 30% of total cell population. However, both KRT-5 (Fig.5.1b and c away from wound) and KRT-14 positive cells (Fig.5.1e away from wound) were hard to detect. This was not entirely surprising, as Rock and colleagues (2009) struggled with a similar problem *in vivo*, reporting that expression from the KRT-14 promoter could be easily detected during epithelial repair, but was very low in homeostatic tissues. Similarly, injury boosted the expression of KRT-5 (Rock *et al*. 2009). Based on these observations, I decide to injure mature MTEC cultures (by scratching) to confirm that the Basal cell population was still present. Indeed, both KRT-5 (Fig.5.1d) and KRT-14 (Fig.5.1e) staining was boosted near the wound. I did not directly test for the presence of Secretory cells. The cilia of the Cilliated cells were visible under a light microscope, and also detected by staining against Acetylated Tubulin-α (Fig.5.1e). In this way, I confirmed the presence of both stem and differentiated cells in my MTEC cultures.

5.3. **Mild elevation of p53 signalling is sufficient to trigger competition in primary MTEC cultures**

To test for mechanical p53 competition in MTEC cultures, I adapted the method previously used to trigger competition between MDCK cells. Namely, to create differences in p53 signalling across cells, I used a chemical activator (Nutlin-3) to elevate p53 in normal cells, and confronted them with p53-null mutants. As before, it was key to only mildly activate p53 signalling in the normal cells, to avoid cell-autonomous death. For this purpose I tested a range of Nutlin-3 concentrations on monocultures of MTECs. By live imaging, I detected little cell death at 25 μM, and most cells died within five days at 40 μM (not shown). For competition experiments, I selected 17.5 μM as a safe concentration, which did not trigger cell-autonomous death even considering culture-to-culture variability. I isolated normal cells from mice carrying a Tomato nuclear label

(*Gt(ROSA)26Sor*$^{tm1(CAG\text{-}tdTomato*,\text{-}EGFP*)Ees}$). This nuclear label allows one to conveniently follow the number and fate of loser cells in competition assays. I isolated p53-null cells from *Trp53*^{tm1tyj} mice, a strain that has 40% of the coding sequences of p53 replaced with a neomycin cassette. The cells were mixed at the time of plating and formed clones with limited intermixing (Fig.2a). Nutlin-3 was added to mature, confluent cultures.

As control for competition assays, I mixed unlabelled and Tomato-labelled MTECs (Fig.5.2a). Like *in vivo* (Rock *et al*. 2009), under normal conditions, confluent MTEC monolayers showed little proliferation or change in cell density (Fig.5.2a,b, compare first and second time points). Interestingly however, mild elevation of p53 (by Nutlin-3 treatment) resulted in a 26% average reduction in cell density, accompanied by cell extrusion, suggesting acquired hypersensitivity to crowding (Fig.5.2a,b, compare before and after Nutlin-3 addition). Similarly to MDCK cultures, co-culture of wild-type (Tomato) and p53-null cells did not result in competition in absence of an external p53 activator (Fig.5.2c,d, compare first and second time points). However, again like in MDCK cultures, addition of Nutlin-3 induced robust cell competition, causing the number of wild-type (Tomato) cells to plummet within six days to about 17% of their starting number, with pronounced cell death and fragmentation observed by live imaging (Fig.5.2c,d). Thus, mild p53 elevation is sufficient to induce crowding hypersensitivity and competition in MTEC cultures.

To exclude the possibility of mutation- of strain-specific results, I conduced a rescue experiment using the p53^{ER} (Trp53^{tm1Gev}) strain. In p53^{ER} mice, the endogenous p53 has been replaced with a p53 fusion protein whose function depends on 4-hydroxytamoxifen (4-OHT). In absence of 4-OHT, p53 is not expressed, while addition of 4-OHT restores p53 to physiological levels (Christophorou *et al*. 2005). As expected, in absence of 4-OHT, unlabelled p53^{ER} cells efficiently out-competed Tomato-labelled normal cells (Fig.5.3a). Strikingly, cell density within the Tomato clones increased after the addition of Nutlin-3, suggesting active compaction of the loser clones

(Fig.5.3a). When p53 levels were restored by 4-OHT, competition did not occur (Fig.5.3b), confirming that this phenomenon depends of p53 signalling.

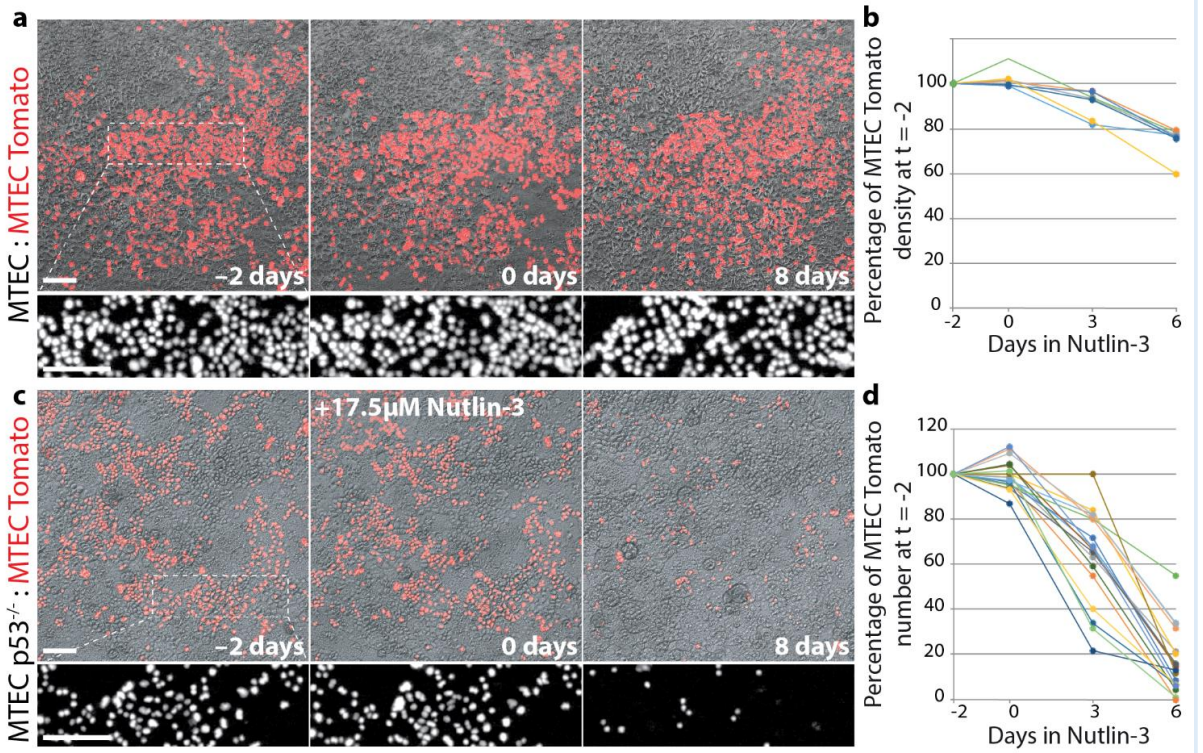


Figure 5.2. Activation of p53 is sufficient to trigger competition in MTEC primary culture.

(**a**) Stills from time-lapse movies of co-cultures of unlabelled and Tomato-labelled wild-type (WT) MTECs. Nutlin-3 (17.5 μM) was added at t=0. (**b**) Time-resolved cell density measurement from movies as in **a**, of WT MTECs before and after Nutlin-3 (17.5mM) addition. (**c**) Stills from time-lapse movies of co-cultures of unlabelled $p53^{-/-}$ and Tomato-labelled WT MTECs. Nutlin-3 (17.5 μM) was added at t=0. (**d**) Time-resolved measurement of cell number from movies as in **c**, of WT MTECs before and after Nutlin-3 (17.5mM) addition. Scale bars = 100 μm.

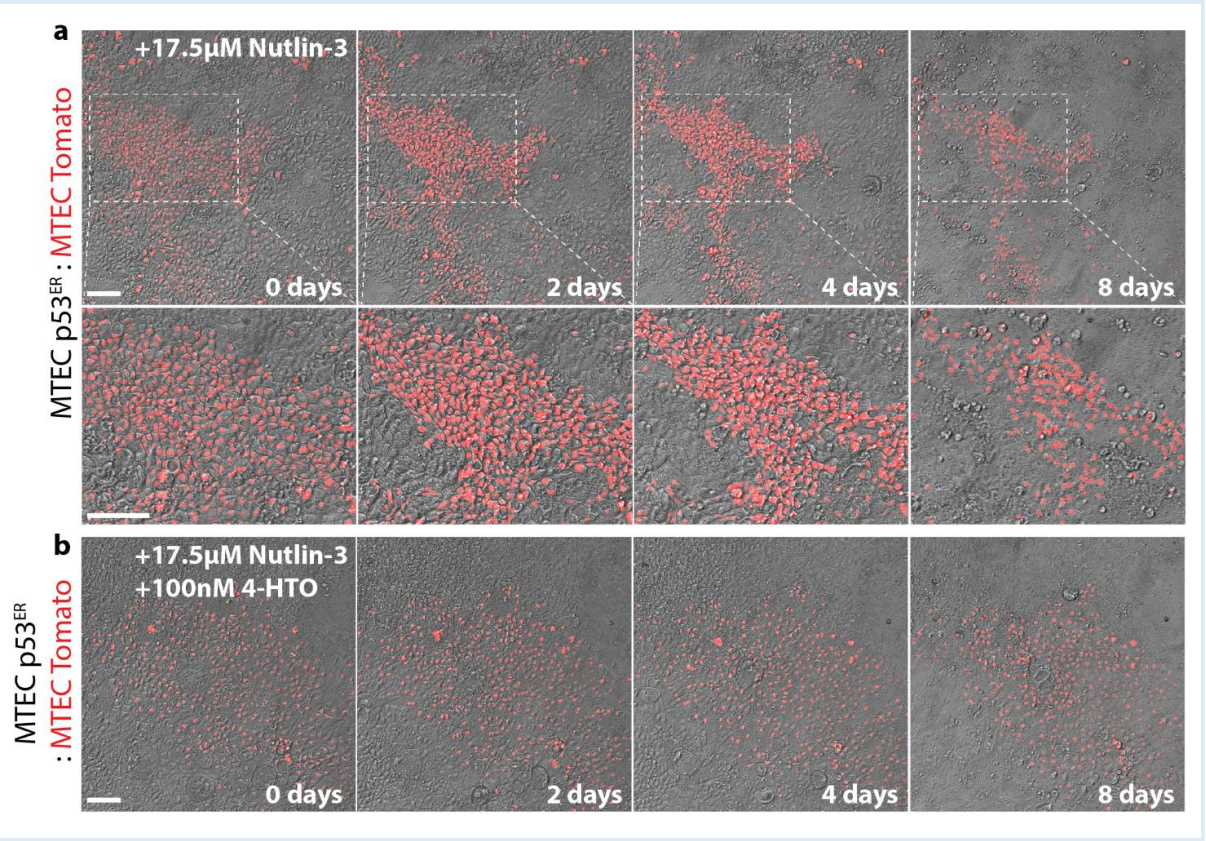


Figure 5.3. Competition boosts cell density in p53-high clones in MTEC culture. Stills from time-lapse movies of co-cultures of Tomato-labelled WT cells and of unlabelled $p53^{ER}$ cells, grown in absence (**a**, $p53^{KO}$) or presence (**b**, physiological levels of p53) of 4-hydroxytamoxifen (4-OHT). Nutlin-3 (17.5 μM) ± 4-OHT was added at t = 0. Scale bars = 100 μm.

5.4. **Discussion**

5.4.1. Mechanical p53 competition is conserved between MDCK and MTEC epithelial cultures

Successful establishment of a p53 mechanical competition model in MTEC culture confirms that this phenomenon is not specific to MDCK cells, but instead may have wide implication for understanding epithelial and cancer biology. Several aspects of MDCK competition were also observed in MTECs, suggesting a common mechanism for the elimination of loser cells: (1) Cells with relatively higher p53 levels become losers. (2) Mild activation of p53 in normal cells induces hypersensitivity to crowding. (3) During competition,

cell density within loser clones increases. It would be now interesting to see, whether the role of other molecules involved in mechanical competition, such as p38 and ROCK, are also conserved between MDCK and MTEC competition models.

The MTEC competition model presents a number of technical advantages but also limitations, compared to MDCK culture. The main disadvantage, other than the fact that MTECs cannot be as easily passaged and expanded as MDCK cells, is that not all experimental methods suitable for MDCKs can be easily adapted to MTECs. For instance, to receive nutrients from underneath, MTECs are normally grown on a porous membrane, and such cannot be easily used in the stretch-release experiments. For this reason, I was unable to directly test whether mechanical compression is sufficient to kill loser MTECs. Among chief advantages of MTECs over MDCKs is that MTEC epithelia can be stably maintained at confluence. In the established MDCK culture competition models, the cells never cease to proliferate and in time begin to form 3D cysts. Therefore, competition can only be conveniently modelled in sub-confluent MDCK cultures, and hence only for a relatively short time. Experiments in MTEC cultures have no such time limit and can be carried in the more physiological environment of a confluent, homeostatic culture. Another advantage of MTECs of MDCKs is related to the specie of origin: many more tools are available for mouse that for dog systems. In conclusion, the very differences between MDCK and MTEC competition models may be their main advantage, as the use of both systems may allow to answer questions which could not be easily addressed in either system alone.

5.4.2. MTEC culture provides an *in vivo*-like model to study competition in an adult epithelium

To the best of my knowledge, the MTEC culture is the first primary, *in vivo*-like culture system used to model competition in an adult epithelium. Until now, most studies investigated competition in simple epithelia composed of

only one type of cells, with the notable exception of a recent study in the more complex epithelium of *Drosophila* gut, also from our lab (Kolahgar *et al*. 2015). In the MTEC system, we will be now able to investigate the interactions of stem and differentiated cells during mammalian competition. In time, the MTEC culture could be also used to attempt to model competition triggered by mutations other than p53.

An interesting observation stems from the study on mechanical p53 competition in MTECs and MDCK cells, as well as p53 competition in the bone marrow (Bondar & Medzhitov 2010; Marusyk *et al*. 2010). In each case an external activator of p53 signalling (Nutlin-3, UV irradiation) was required to generate a discontinuity in p53 signalling and to trigger competition. This observation may be particularly important for understanding the role of p53 competition in the trachea. Cigarette smoke and other airborne stressors are major contributors to lung cancer and other diseases of the respiratory system. It is therefore conceivable that, with their direct access to external stressors, trachea and the rest of the respiratory system may be particularly susceptible to p53 competition.

Chapter 6. COMPACTION LEADS TO ELEVATION OF P38 VIA ROCK

6.1. Introduction

Having established that elevation of p53 is induced by compaction and required for the elimination of *scrib*$^{KD+TET}$ cells, we next wondered how mechanical stress might lead to p53 activation. A potential candidate was p38 signalling, as p38 signalling in *scrib*$^{KD+TET}$ cells is required for their elimination by competition (Norman *et al*. 2012). Moreover, p38 has been previously reported to activate p53 signalling, e.g. in response to UV irradiation in the mouse epidermal JB6 Cl41 cell line and in breast epithelial MCF-7 cells (Huang *et al*. 1999; Bulavin *et al*. 1999; Cuadrado & Nebreda 2010). Importantly, p38 has been also implicated in response to mechanical stress. Application of mechanical pressure by placing weights on human keratinocyte HaCaT cultures triggered rapid phosphorylation and thus activation of p38 (Hofmann *et al*., 2004). Laura Wagstaff from our group employed the stretch-release assay to investigate the effect of compaction on p38 signalling in *scrib*$^{KD+TET}$ cells and found that compression alone causes an increase in active phosphorylated (T180/Y182) p38 in *scrib*$^{KD+TET}$ cells. Moreover, the upregulation of p53 in competing *scrib*$^{KD+TET}$ cells was reduced in presence of the p38 inhibitor SB202190 (Wagstaff *et al*. 2016) placing p38 upstream of p53 in death by competition.

6.2. Inhibition of p38 signalling protects *scribble*-deficient cells from compaction-induced death

To directly test whether compression triggers apoptosis via p38 I employed the stretch-release system by culturing *scrib*$^{KD+TET}$ cells at sub-confluence or at confluence with or without the p38 inhibitor SB202190. Inhibition of p38 had no appreciable effect on the viability of uncompressed (sub-

confluent) cultures (1.63±1.80% apoptotic events/field for DMSO control vs 0.92±1.44% for SB202190; Fig.7.1) but significantly reduced cell death in compressed (confluent) cultures (7.78±1.57% apoptotic events/field for DMSO control vs 4.02±1.33% for SB202190; Fig.7.1). Together, these results suggest that p38 signalling triggers apoptosis of *scrib*$^{KD+TET}$ cells selectively in response to mechanical compression. As a caveat, a mild effect on the background level of cell death (uncompressed cultures) might have been overlooked due to the sensitivity of the assay. Moreover, the rescue of cell viability at high density was not complete. This might have been either due to incomplete inhibition of p38 signalling, or an indication that an additional, parallel pathway triggers cell death in response to compression. Nevertheless, the results conclusively demonstrated a role of p38 in induction of apoptosis upon compression.

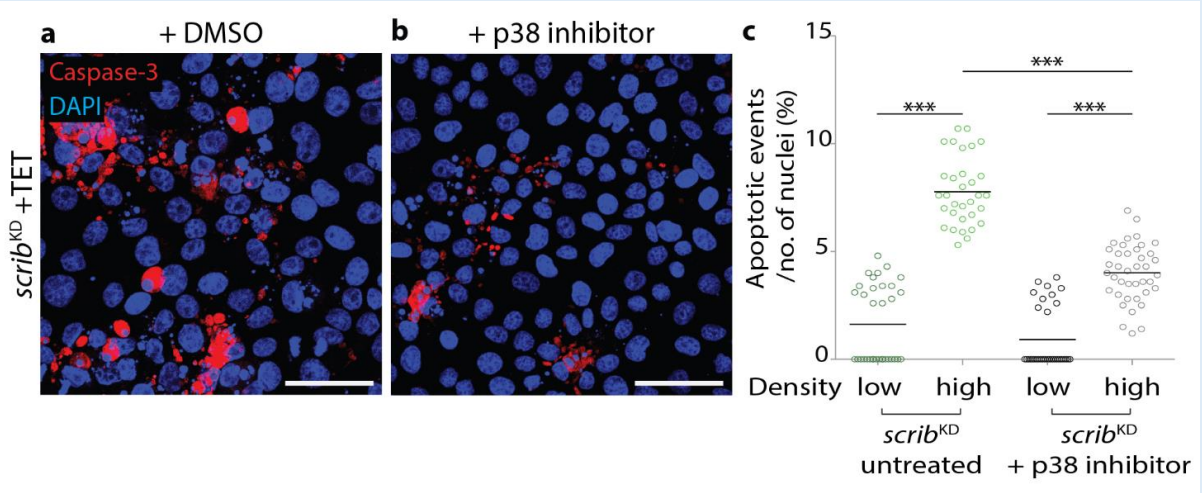


Figure 6.1. Compression triggers death of *scribble*-deficient cells by activating p38. *scrib*$^{KD+TET}$ cells were cultured at two densities (confluence and sub-confluence) on a stretched PDMS membrane in presence of a p38 inhibitor (SB202190) or mock treatment (DMSO). The samples were fixed five hours after stretch release. Cell death was detected by immunofluorescent staining against activated Caspase-3. (**a-b**) Representative confocal images of *scrib*$^{KD+TET}$ low density (left panels) and high density (right panels) cultures treated with DMSO (**a**) or SB202190 (**b**). (**c**) Quantification of cell death (activated Caspase-3) for *scrib*$^{KD+TET}$ *vs scrib*$^{KD+TET}$ *p53*$^{-/-}$ cells +/- compression; black bars = mean. *** p<0.0005 by KS test. Scale bars = 50 µm.

6.3. **Compaction activates ROCK in *scribble*-deficient cells**

Having established that p38 contributes to activation of p53 in *scribble* competition, we attempted to unravel other signalling molecules involved in compaction-induced death of *scrib*$^{KD+TET}$ cells. Earlier studies on competition in MDCK culture reported elevation of Myosin-II selectively in mutant (Ras- or Src-transformed) cells surrounded by normal cells (Hogan *et al*., 2009; Kajita *et al*., 2010). Based on these results, and considering the mechanical aspect of *scribble* competition, we decided to investigate whether compression of *scrib*$^{KD+TET}$ cells induces changes in the acto-Myosin cytoskeleton. Indeed, immunofluorescent staining revealed an accumulation of both cortical Actin (by Phalloidin staining; Fig.6.2c) and active phosphorylated-Myosin (P-Myosin) in compacted *scrib*$^{KD+TET}$ cells during competition (Fig.6.2a). Since the cytoskeletal regulator Rho kinase (ROCK) is one of the main kinases responsible for Myosin phosphorylation, we decided to test if it is activated in compacted *scrib*$^{KD+TET}$ cells. Indeed, P-Myosin upregulation upon compaction was reduced in the presence of a ROCK inhibitor (Fig.6.2b). Moreover, phosphorylation of another target of ROCK, the Myosin phosphatase target subunit 1 (Wilkinson *et al*., 2005; MYPT1) was elevated in compacted *scrib*$^{KD+TET}$ cells (Fig.6.2d). Together, these results indicate that ROCK is activated in compacted *scrib*$^{KD+TET}$ cells.

6.4. **ROCK signalling in *scribble*-deficient cells is required for their elimination by mechanical competition**

Having established that ROCK signalling is elevated in compacted loser cells I then asked whether it is required for their elimination. For this purpose I live-imaged co-cultures of wild-type and *scrib*^{KD} cells grown in presence of tetracycline, with or without the ROCK inhibitor Y27632. Inhibition of ROCK did not prevent compaction of *scrib*$^{KD+TET}$ cells surrounded by normal cells, but it did block their elimination (Fig.6.3a). The frequency of apoptotic cells

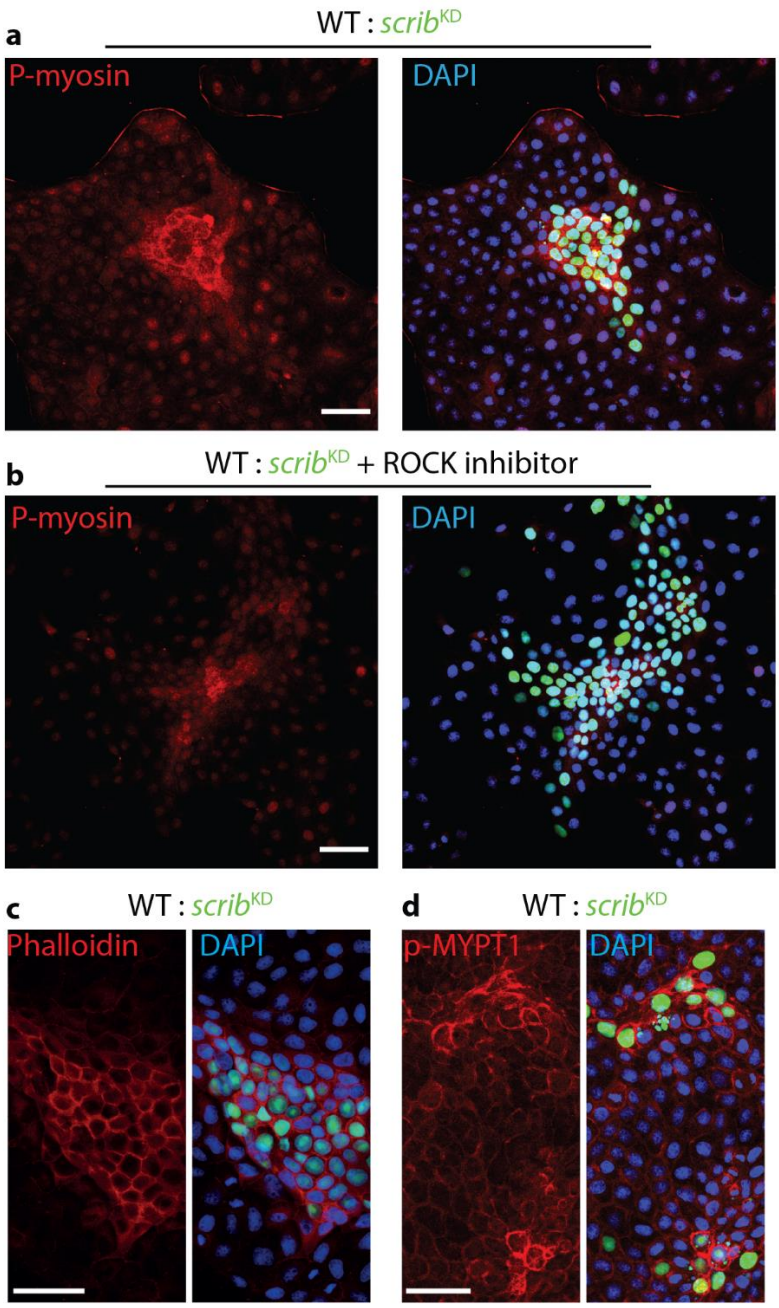


Figure 6.2. ROCK is activated in compacted *scribble*-deficient clones during competition.

A number of targets of ROCK are upregulated in compacted *scrib*$^{KD+TET}$ cells during competition. (**a**-**d**) Maximal projections of confocal images from co-cultures of unlabelled WT and GFP-labelled *scrib*^{KD} cells grown in presence of tetracycline. (**a**-**b**) Myosin-II is phosphorylated (P-Myosin) in compacted *scrib*$^{KD+TET}$ clones (**a**) and this phosphorylation is reduced in presence of a ROCK inhibitor (Y27632). (**c**-**d**) F-Actin (phalloidin-stained, **c**) and phosphorylated MYPT1 (p-MYPT1, **d**) are elevated in compacted *scrib*$^{KD+TET}$ clones. Scale bars = 50 µm.

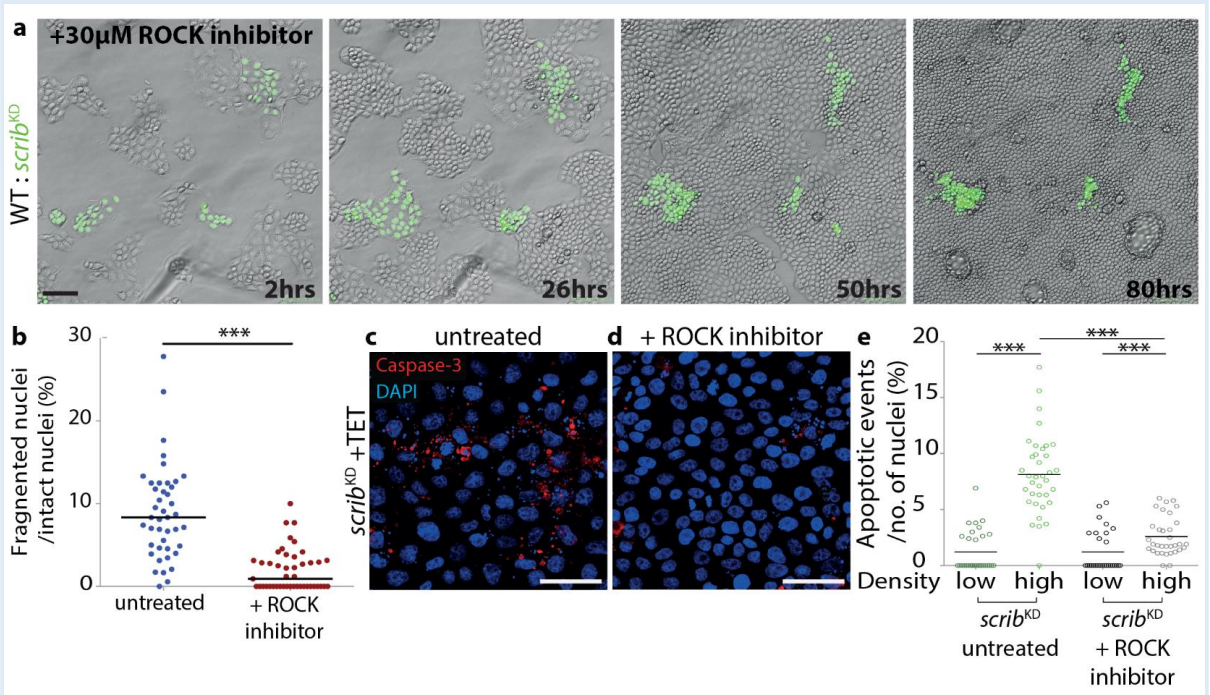


Figure 6.3. Activation of ROCK in *scribble*-deficient cells is required for their elimination.

(**a**) Stills from time-lapse movies of WT and *scrib*^{KD} cells co-cultured in the presence of tetracycline and a ROCK inhibitor (Y27632); scale bar = 100 μm. (**b**) Quantification of the fraction of dying cells in GFP-labelled *scrib*$^{KD+TET}$ clones, measured as the number of fragmented GFP-labelled nuclei per number of intact GFP-labelled nuclei. Each dot represents a single clone. Black bars = mean; ***; p<0.0005 by KS test. (**c-e**) *scrib*$^{KD+TET}$ cells were cultured at two densities (confluence and sub-confluence) on a stretched PDMS membrane in presence or absence of the ROCK inhibitor. The samples were fixed five hours after stretch release. Cell death was detected by immunofluorescent staining against activated Caspase-3. (**c-d**) Representative confocal images of *scrib*$^{KD+TET}$ low density (left panels) and high density (right panels) cultures grown in absence (**c**) or presence of the ROCK inhibitor (**d**). (**e**) Quantification of cell death (activated Caspase-3) for images as in (**c**,**d**). Black bars = mean. ***; p<0.0005 by KS test. Scale bars = 50 μm.

(fragmented nuclei) detected within *scrib*$^{KD+TET}$ clones surrounded by wild-type cells was significantly reduced in presence of the inhibitor (Fig6.3.b), suggesting that compaction triggers death via activation of ROCK signalling. The rescue was not complete, possibly either due to an incomplete inhibition of ROCK signalling or due to another pathway signalling in parallel to ROCK.

A disadvantage of using chemical inhibitors in competition assays is that they may affect both the loser and the winner cell population. To eliminate

the possibility that *scrib*$^{KD+TET}$ clones were rescued due to inhibition of ROCK signalling in the normal cells, I employed the stretch-release system to directly test the function of ROCK in compression-induced death of the loser cells. I plated *scrib*$^{KD+TET}$ at sub-confluence (uncompressed culture) and at confluence (compressed culture). To assure that the initial cell numbers were comparable between treated and untreated cells, and not affected by any potential effect of ROCK on cell growth, I cultured the *scrib*$^{KD+TET}$ in absence of additional treatment, and only added the inhibitor one hour before compression. As in the case of p38 inhibition, the background level of death (uncompressed cultures) was not significantly affected by ROCK inhibition (1.21±1.82% apoptotic events/field for control vs 1.22±1.84% for Y27632; Fig.6.3c-e). This may be explained either by a lack of effect of ROCK on cell-autonomous viability of *scrib*$^{KD+TET}$ cells, or by insufficient sensitivity of the assay. In contrast, inhibition of ROCK massively reduced death of the mutant cells upon compression (8.14±3.51% apoptotic events/field for control vs 2.58±1.72% for Y27632; Fig.6.3c-e), suggesting that ROCK plays a major role in compaction induced apoptosis of *scrib*$^{KD+TET}$ cells.

6.5. **Compression activates p38 signalling via ROCK**

Knowing that both p38 and ROCK are activated in compressed *scrib*$^{KD+TET}$ cells and required for their elimination I then asked whether they function independently or as components of a single signalling cascade. Since ROCK reportedly activates p38 signalling upon mechanical compression of bovine cartilage (Nakagawa *et al*. 2012), I decided to first test whether ROCK signals upstream of p38 during mechanical cell competition. For this purpose I again employed the stretch-release system. The *scrib*$^{KD+TET}$ cells were cultured in presence or absence of the ROCK inhibitor, but unlike in the previously-described experiments, they were only grown at confluence. Activation of p38 was assessed by immunofluorescence, by measuring the level of phosphorylated (active) p38 in the nuclei. Inhibition of ROCK

moderately, but significantly reduced activation of p38 upon compaction (Fig.6.4). As a caveat, the effect of ROCK on p38 activation was more pronounced upon milder compression than that applied in the previous assays. This may suggest that while ROCK signals upstream of p38, upon extreme compression, other parallel mechanisms compensate for the absence of ROCK to activate p38.

6.6. **Discussion**

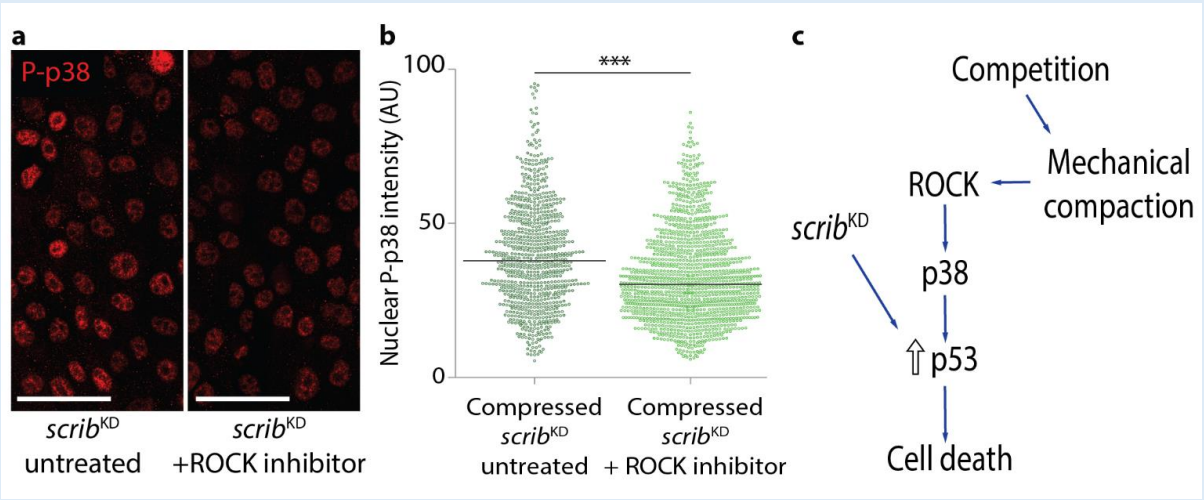


Figure 6.4. Compaction activates p38 via ROCK.
(**a-b**) *scrib*$^{KD+TET}$ cells were cultured at confluence on a stretched PDMS membrane with tetracycline and in presence or absence of a ROCK inhibitor (Y27632). The samples were fixed 1.5 hours after stretch release and immunostained against phosphorylated (active) p38 (P-p38). (**a**) Representative confocal images of untreated (left panel) *scrib*$^{KD+TET}$ cultures and cultures treated with ROCK inhibitor (right panel). (**b**) Quantification of single cell nuclear P-p38 intensity from images as in (**a**). Black bars = median. *** $p<0.0005$ by KS test. Scale bars = 50 µm. AU = arbitrary units. (**c**) Model of mechanical cell competition between *scrib*$^{KD+TET}$ and wild-type cells.

6.6.1. Compaction activates p53 via ROCK and p38

In this chapter I described the discovery of a signalling cascade where upon compaction ROCK activates p38 which in turn boosts p53 in *scrib*$^{KD+TET}$ cells, triggering their apoptosis (Fig.6.4c). Together, based on data described in this and previous chapters, I propose a model in with *scrib*$^{KD+TET}$ cells cell-

autonomously mildly up-regulate p53 signalling, and this up-regulation renders them hypersensitive to density. Compaction, via activation of ROCK and the stress kinase p38, leads to further elevation of p53, causing cell death. In the future, it would be interesting to test whether p38 and ROCK are also involved in the elimination of loser cells in p53 mechanical competition, as better understanding of the molecular basis of p53 competition might yield novel therapies for cancer.

6.6.2. A novel role of ROCK in mammalian cell competition

The involvement of ROCK in competition between MDCK cells has been previously reported for out-competition of Ras- and Src-transformed cells (Kajita *et al*. 2014). However, in these models ROCK was activated in the adjacent normal cells, where it was required to generate the contractile force that leads to the epical extrusion of the mutants. Similarly, activation of ROCK in the surrounding cells was required for apical extrusion of apoptotic MDCK cells and of supernumerary MDCK cells during a stretch-release assay, where it signalled downstream of Rho, promoting formation and contraction of an acto-Myosin ring around the extruded cell, which pushed them out of the epithelium. (Eisenhoffer *et al*. 2012; Gu *et al*. 2011). Although we did not observe the formation of an actoMyosin ring arround *scrib*$^{KD+TET}$ clones, it might have been obscured by the elevation of F-actin within those clones. An acto-Myosin ring might have been also harder to detect since, unlike all of the above authors, we studied the elimination of multicellular clones, and not individual cells. Nevertheless, the stretch-release experiments conclusively proved that it is the ROCK signalling in the mutant cells, and not in the normal cells, that triggered loser death. This leaves the possibility that in addition to its function in the loser clones, activation of ROCK in winners contributes to the compaction of *scrib*$^{KD+TET}$ clones. However, live imaging did not suggest that this is the case (Fig.3a), as the density within the mutant clones still exceeded that in the surrounding normal cells.

Future studies on the involvement of ROCK in mechanical competition could address two questions: (1) How is ROCK activated upon compaction and (2) How does ROCK activate p38 and p53. (1) An obvious candidate for a ROCK activator would be the Rho kinase. Unfortunately, preliminary experiments (data not shown) indicated that even short term inhibition of Rho is toxic to *scrib*$^{KD+TET}$ cells, and a more involved approach, such as the use of a Rho-ROCK FRET probe would have to be considered. (2) In this chapter I proposed that ROCK activates p53 via p38. However, I did not address the question on how does ROCK regulate p38. The best known direct target of ROCK is the light chain of Myosin (Amano *et al*. 2010). However, earlier studies by Norman and colleagues (Norman *et al*. 2012) indicated that Blebbistatin, a Myosin inhibitor, did not block apoptosis of *scrib*$^{KD+TET}$ cells during competition, indicating that another target of ROCK may be involved in competition. In addition to establishing how ROCK regulates p38, future experiments may reveal an additional, p38-independent, connection between ROCK and p53. Such questions would be more easily addressed using genetic tools, rather than the chemical inhibitors described in this chapter, as they would assure complete inhibition of the components of the pathway.

Chapter 7. FOCAL ADHESIONS AND SRC SIGNALLING IN MECHANICAL CELL COMPETITION

7.1. Introduction

The most dramatic feature of mechanical competition is the drastic change is shape of losers from flattened to compacted. I decided to investigate how this change in morphology may contribute to competition. As described before, there are two main aspects to the macroscopic changes in loser cell shape: (1) As a consequence of increased cell density, the area of adhesion to the extracellular substrate decreases over four-fold for each cell. This is like to affect the numbers and distribution of cell-substrate adhesion sites. (2) Simultaneously, the cells become taller, which likely affects the composition and functioning of cell-cell adhesions. Since both cell-substrate and cell-cell adhesions are key to interpreting environmental clues, and both have been implicated in responses to mechanical stimuli (Jaalouk & Lammerding 2009), either is likely to be involved in compaction-induced death.

In this chapter, I describe exploratory experiments designed to address the role of changes in cell shape, and in particular changes in cell-substrate adhesions, in connection to cell competition. These studies are by no means comprehensive or completed, but provide interesting leads and observations. All experiments were carried on MDCK cells in the *scribble* and/or in the p53 competition system, and were conducted either by myself (p53 competition) or together with an undergraduate student under my supervision, Anna Klucnika (*scribble* competition).

We first addressed the question of how a change in the substrate-adhesion area may affect the viability of loser cells, concentrating on integrin signalling. Integrins are transmembrane heterodimers that couple

extracellular matrix (ECM) to the cytoskeleton. They play a double role of anchoring the cell to the substrate, and of transducing signals from the ECM to the cells. Importantly, when deprived of integrin anchorage, normal epithelial cells undergo detachment-induced apoptosis, termed anoikis (Taddei *et al*. 2012). Integrins act as part of large protein complexes, known as focal adhesions (FAs). Among the components of FAs involved in survival signalling (Vachon 2011), is the proto-oncogene tyrosine-protein kinase Src, which has been previously implicated in competition (Kajita *et al*. 2010).

Src plays diverse function in survival, cell differentiation, proliferation, migration and shape regulation (Parsons & Parsons 2004) and can be found in multiple cell compartments. As it is found both in FAs and in the lateral membranes, where it promotes E-cadherin turnover (Parsons & Parsons 2004), Src is a likely candidate for a protein regulated by the compression-induced change of shape.

7.1.1. Increased focal adhesion assembly protects *scribble*-deficient cells from out-competition

Loser cell death correlates with a drastic reduction in cell-substrate adhesion area. I hypothesized that the number of FAs may correspondingly drop, and that the resulting reduction in survival signalling triggers death via an anoikis-like mechanism. I further predicted that *scrib*$^{KD+TET}$ cells flatten in homotypic cultures to maximise the integrin survival signalling, but that this additional signalling is lost when the cells are compacted during competition. To test this hypothesis, I first compared the distribution of FAs in wild-type cells and in un-compressed and compressed *scrib*$^{KD+TET}$ cells by staining against the FA-component vinculin (Fig.7.1a-b), and found that the distribution of vinculin differed between all three conditions. While large FAs anchored stress fibres (Phalloidin staining) in un-compressed *scrib*$^{KD+TET}$ cells (Fig.7.1a), FAs in wild-type cells (Fig.7.1b, yellow stars in the right panel) were much smaller and more evenly distributed, suggesting a cell-

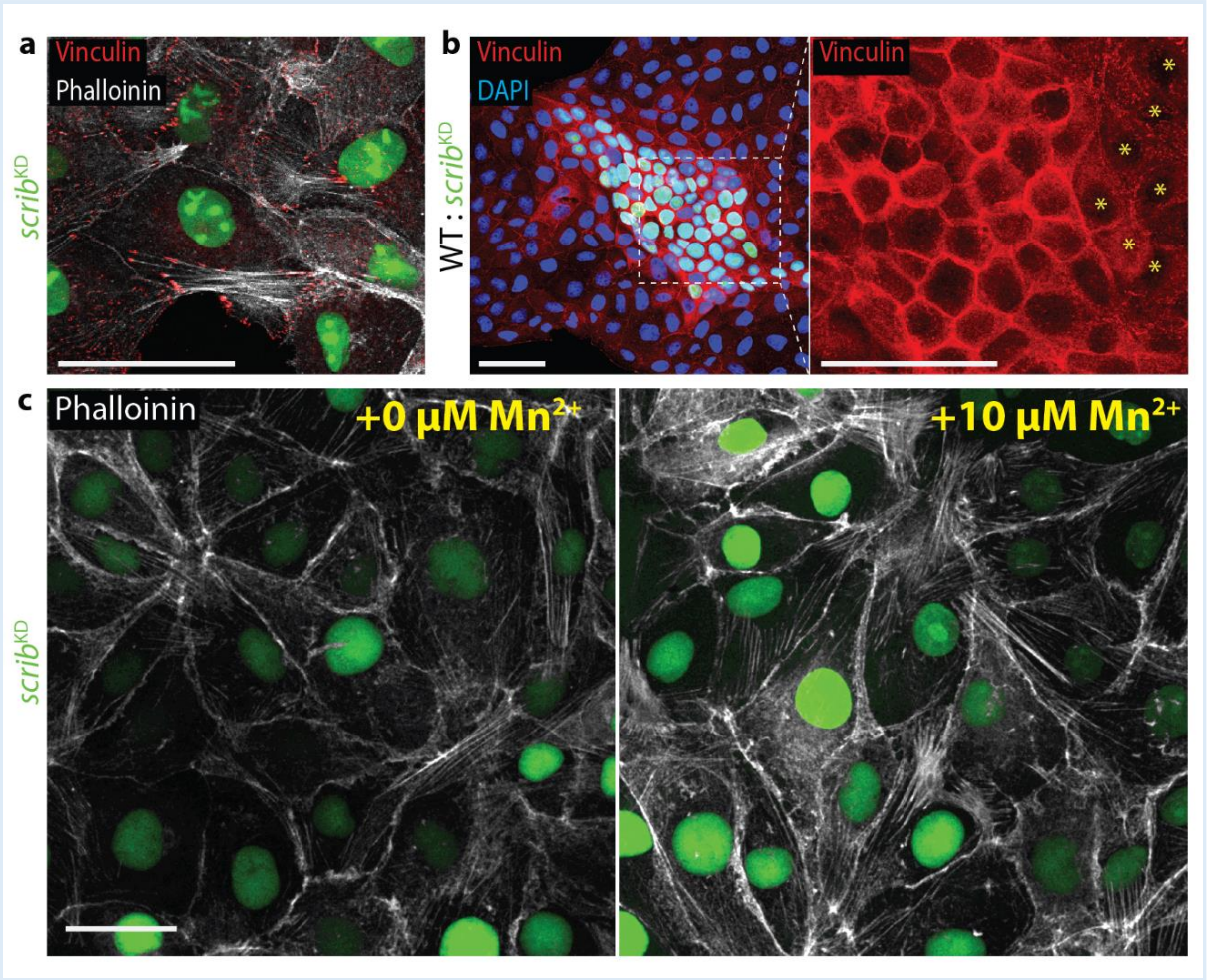


Figure 7.1. Focal adhesions and stress fibres in *scribble*-deficient mechanical losers. (**a**,**b**) Vinculin localises to large focal adhesions at ends of stress fibres (Phalloidin) in homotypic cultures of *scib*^{KD} cells (**a**), but translocated to cell-cell junctions in *scrib*^{KD} clones compacted by WT cells (**b**). Yellow stars in the right panel mark WT cells. (**c**) Integrin-activator Mn^{2+} induces accumulation of stress fibres in homotypic cultures of *scib*^{KD} cells. Scale bars = 50 µm.

autonomous difference in FA signalling even prior to competition. Even more strikingly, in compressed *scrib*$^{KD+TET}$ cells vinculin translocated from FAs to the sites of cell-cell contact, suggesting a change in balance between cell-substrate and cell-cell signalling (Fig.7.1b).

If *scrib*$^{KD+TET}$ cells flatten to maximise integrin signalling, then ectopic activation of integrin signalling should relieve the need for increased substrate-adhesion area, and rescue *scrib*$^{KD+TET}$ cell shape. I decided to test this theory by treating homotypic cultures of *scrib*$^{KD+TET}$ cells with Mn^{2+}, as it has been shown to activate integrins and to promote the formation of FAs

(Dormond *et al*. 2004). In the time-frame of competition assays, at the high concentrations used in previous studies (1 mM and 0.5 mM, Dormond *et al*. 2004; Lee *et al*. 2015) Mn^{2+} was toxic to both wild-type and *scrib*$^{KD+TET}$ cells (Fig.7.2a,b). Therefore, we decided to test the long term effects of a milder dose of Mn^{2+} on MDCK cells. By staining against filamentous actin, we were able to detect an accumulation of stress fibres, which accompanies the formation of FAs in Mn^{2+}-treated samples (Dormond *et al*. 2004), even at 10 μM (Fig.7.1c). Interestingly, 10 μM Mn^{2+} selectively increased the density of pure cultures of *scrib*$^{KD+TET}$ cells (Fig.7.2b,c), while having no appreciable effect on the density of wild-type cells (Fig.7.2a,d), suggesting that *scrib*$^{KD+TET}$ cells may be particularly sensitive to integrin signalling.

Encouraged by the observation that increased integrin signalling reduces the hypersensitivity to compaction of *scrib*$^{KD+TET}$, I then decided to test the effect of Mn^{2+} on competition. At 10 μM Mn^{2+} did not prevent compaction of *scrib*$^{KD+TET}$ cells, but it partially blocked their elimination (Fig.7.2a-b), suggesting that activation of integrin signalling protects *scrib*$^{KD+TET}$ cells by reducing their hypersensitivity to compaction. To confirm that Mn^{2+} blocked competition by affecting the losers and not the winners, we employed the stretch-release assay. *scrib*$^{KD+TET}$ cells were plated at sub-confluence (uncompressed control) and at confluence (compressed cultures) +/- 10 μM Mn^{2+}. Mn^{2+} did not significantly affect cell-autonomous viability of *scrib*$^{KD+TET}$ cells (low density cultures, Fig.7.3e), but substantially blocked apoptosis of compressed cultures (high density cultures, Fig.7.3c-e), suggesting that Mn^{2+} protects *scrib*$^{KD+TET}$ cells specifically from compaction-induced death. Together, the above data argues that activation of integrins protects *scrib*$^{KD+TET}$ cells from out-competition by reducing their hypersensitivity to compaction. It also suggests that *scrib*$^{KD+TET}$ cells are particularly sensitive to the levels of integrin signalling, supporting, but not

conclusively proving, the theory that compaction kills loser cells by reducing the cell-substrate adhesion area.

I attempted to reproduce the above results in the p53 competition model. Preliminary assays did not reveal any substantial effect of 10 μM Mn^{2+} on competition (data not shown). Since the effect of Mn^{2+} on *scrib*$^{KD+TET}$ cells was strongly dose-dependent, and the increase in density only observed for a narrow range of concentrations, p53-high cells might have required a

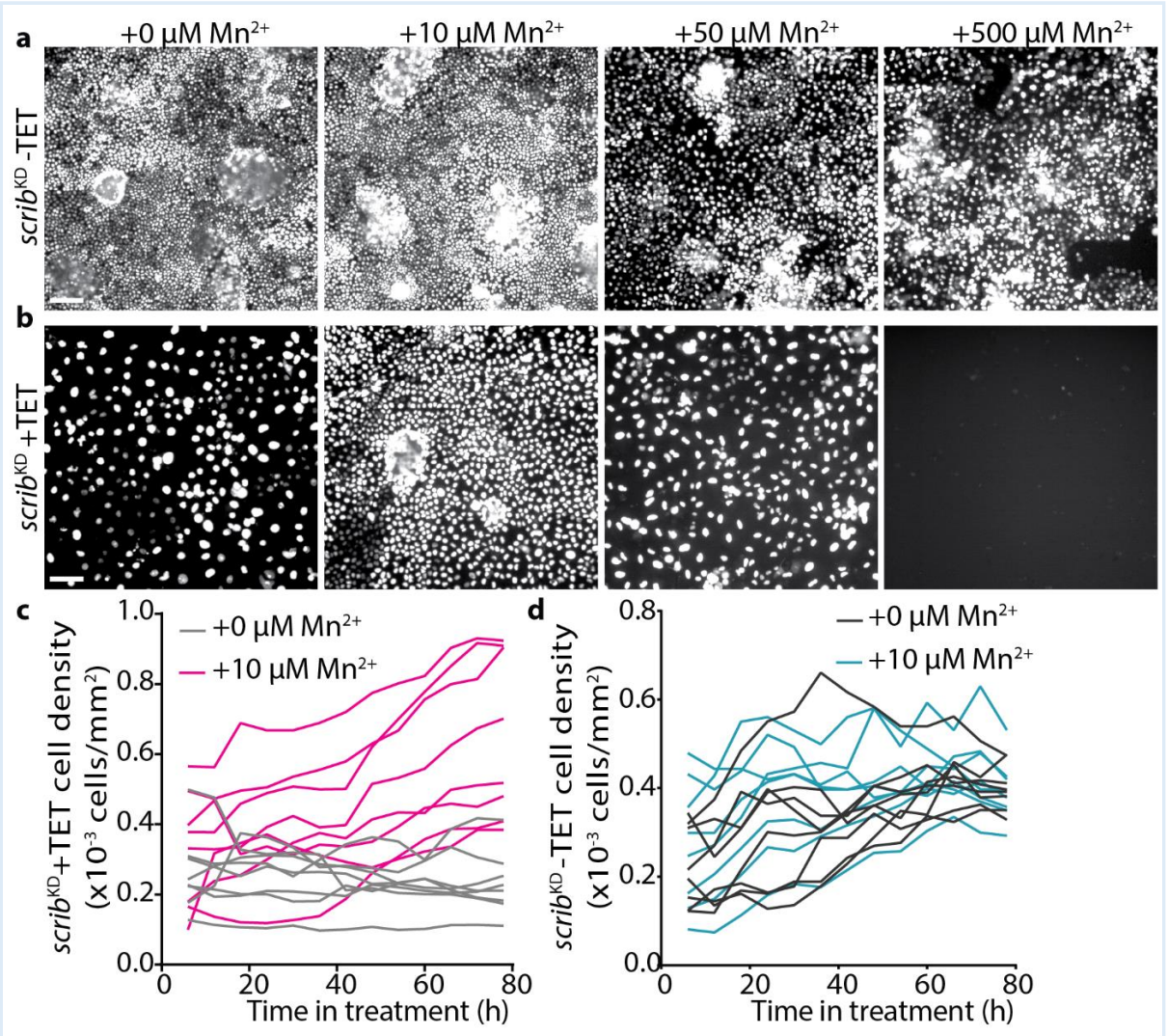


Figure 7.2. Mn^{2+} reduces sensitivity of *scribble*-deficient cells to cell density.
(**a**-**d**) Live-imaging of *scrib*^{KD} cells –TET (wild-type, **a**,**d**), or +TET (*scribble* RNAi, **b**,**c**) treated with Mn^{2+}. (**a**,**b**) Stills from dose-response assay, taken 78 h into treatment, and showing GFP-labelled nuclei of *scrib*^{KD} cells. (**c**,**d**) Quantification of cell density over time +/- 10 μM Mn^{2+} and +TET (**c**) or –TET (**d**), from images as in **b** and **a**, respectively. Each line corresponds to a single imaged field. (**c**) **; P<0.005 by KS test at t=78 h into treatment. (**d**) P = 0.98 by KS test at t=78 h into treatment. Scale bars = 100 μm.

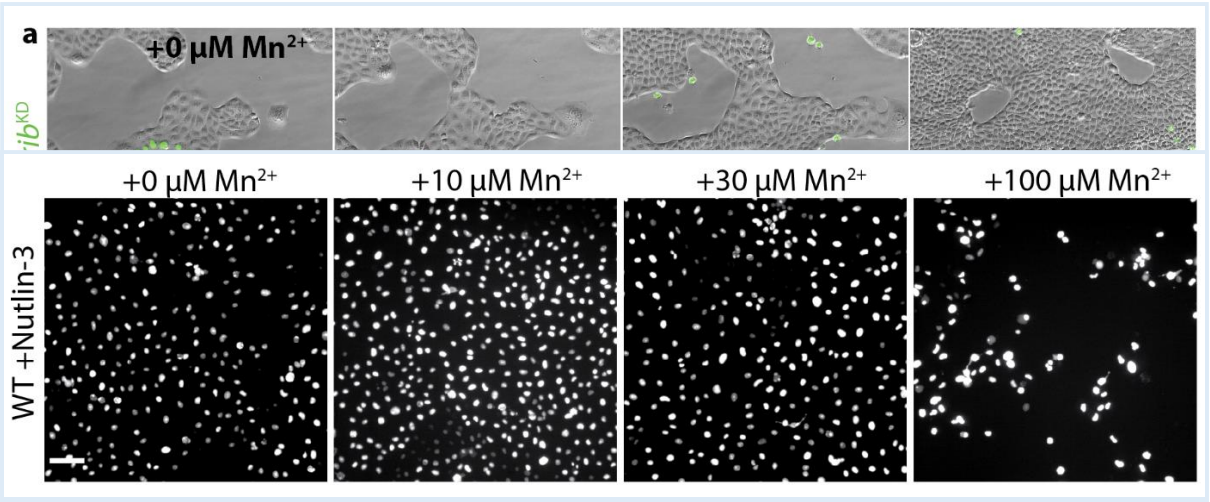


Figure 7.4. Mn^{2+} mildly reduces sensitivity of p53-high cells to cell density.
Dose-response assay testing the effect of Mn^{2+} on WT cells treated with Nutlin-3. Stills from time-lapse taken 84 h into treatment; showing GFP-labelled nuclei. Scale bars = 100 μm.

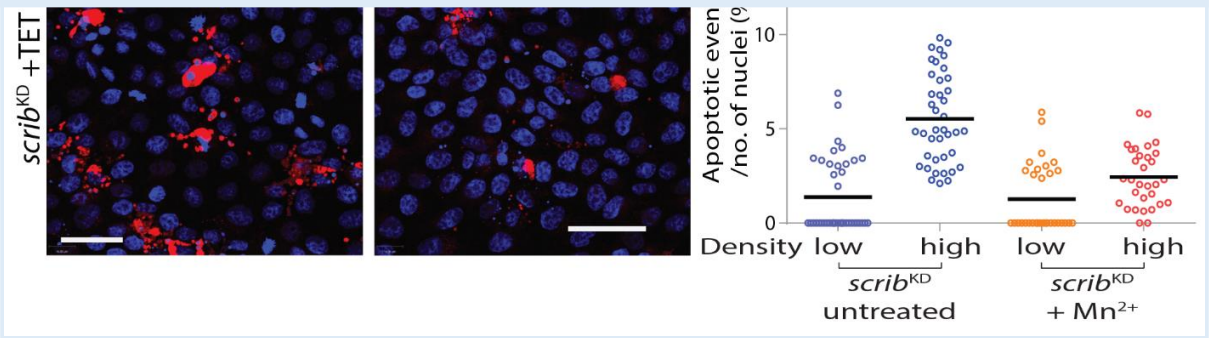


Figure 7.3. Activation of integrin signalling protects *scribble*-deficient cells from mechanical out-competition.
(**a**-**b**) Stills from time-lapse movies of WT and *scrib*^{KD} cell co-cultures in the presence of tetracycline, without (**a**) or with 10 μM Mn^{2+}; scale bars = 100 μm. (**c**-**e**) *scrib*$^{KD+TET}$ cells were cultured at two densities (confluence and sub-confluence) on a stretched PDMS membrane in absence (**c**) or presence (**d**) of 10 μM Mn^{2+}. The samples were fixed five hours after stretch release. Cell death was detected by immunofluorescent staining against activated Caspase-3. Scale bars = 50 μm.

different dose of treatment than *scrib*$^{KD+TET}$ cells. To test this possibility, I treated p53-high cells with a range of concentrations of Mn^{2+} (5, 10, 20, 30, 40, 50, 100, μM), but again only observed an increase in density at 10 μM. The effect on density in p53-high cultures was very mild (Fig.7.4), compared to *scrib*$^{KD+TET}$ cultures, explaining why any potential effect on competition may be harder to detect.

7.2. Src signalling is required for the elimination of p53-high mechanical losers

Continuous activation of Src has been reported to confer both a loser cell status (Kajita *et al*. 2010) and resistance to anoikis (Dohn *et al*. 2009) in the MDCK cells. Therefore, I decided to test whether it is also involved in out-competition of mechanical losers. First, together with Anna Klucnika, I stained against phosphorylate (pY412, active) Src (P-Src). In homotypic cultures of *scrib*$^{KD+TET}$, P-Src localised strongly to the nuclei (Fig.7.5a) and to FAs (Fig.7.5a, see yellow arrows in right panel), and weakly to cell-cell junctions (Fig.7.5a). During competition, FAs could no longer be detected in *scrib*$^{KD+TET}$ clones by P-Src staining (Fig.7.5b-c). Instead, shortly after the onset of compaction, junctional staining increased to above that in the surrounding normal cells (Fig.7.5b). Additionally, as the compaction proceeded, P-Src translocated from nuclei (Fig.7.5c). Together, these changes suggest that P-Src may be involved in competition, and that it may play multiple roles depending on the stage of competition.

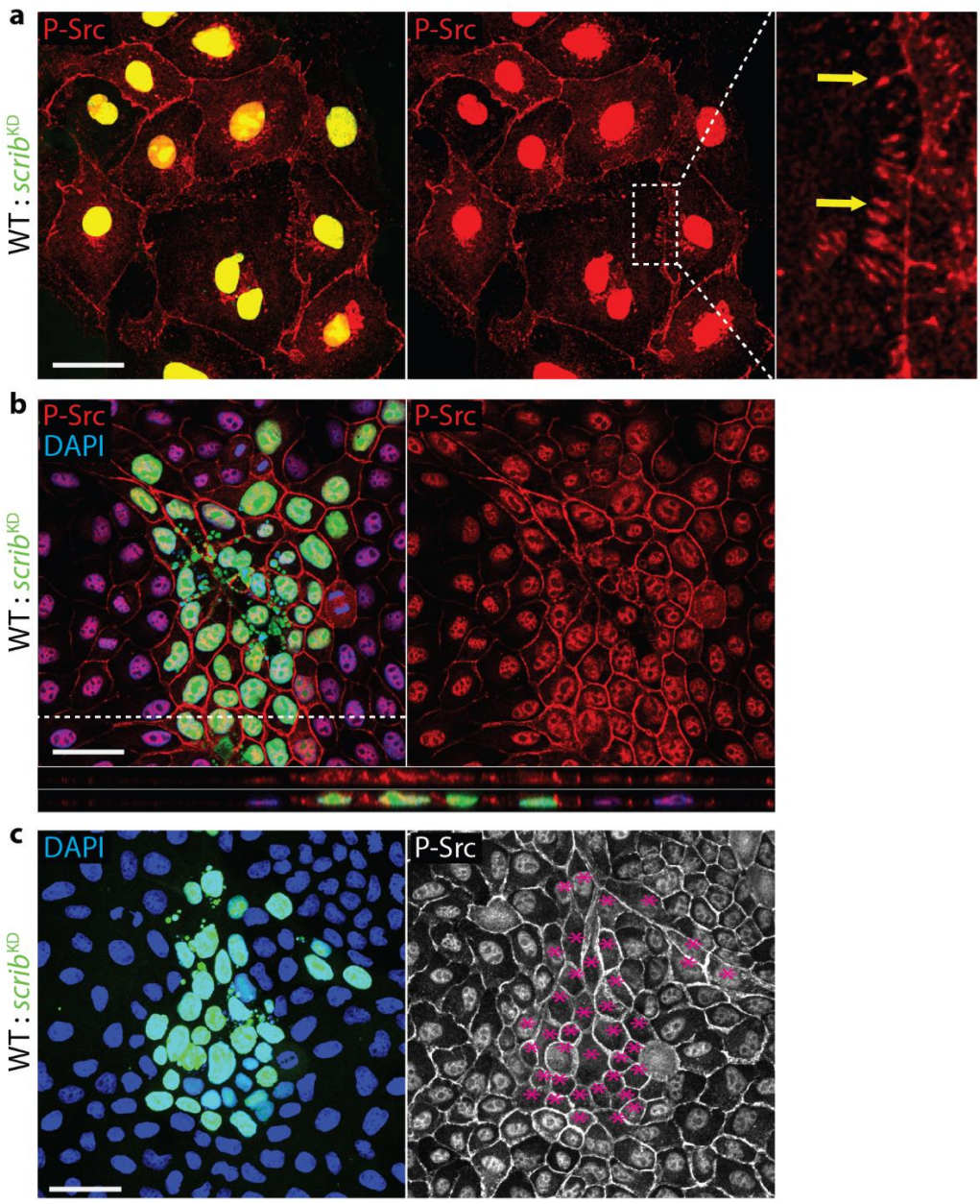


Figure 7.5. Translocation of Src upon compaction of *scribble*-deficient clones.
(**a**) In homotypic (GFP-labelled) *scrib*^{KD} cultures, phosphorylated/active Src (P-Src) localises predominantly to nuclei and focal adhesions (see yellow arrows in right panel), and is also found at cell-cell junctions. (**b**,**c**) In *scrib*^{KD} clones (GFP-labelled) compacted by WT cells (unlabelled), P-Src localises predominantly to cell-cell junctions (**b**), and as compaction progresses, it becomes excluded from nuclei (**c**). (**b**) Single section of a z-stack and a xy section corresponding to white dotted line. (**c**) Stars in right panel mark GFP-labelled *scrib*^{KD} cells. Scale bars = 50 µm.

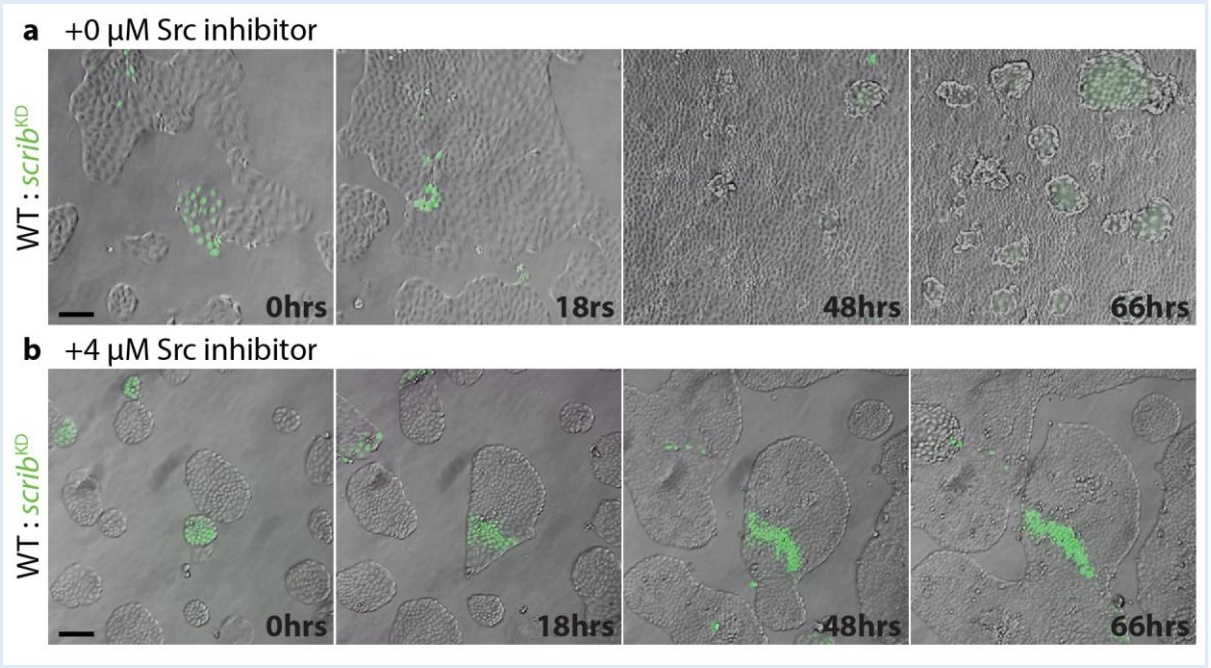


Figure 7.6. Activation of Src is required for elimination of *scribble*-deficient cells.
(**a**-**b**) Stills from time-lapse movies of WT and *scrib*^{KD} cells co-cultured with TET and without (**a**) or with (**b**) a Src inhibitor (PP2); scale bars = 100 µm.

Next, we asked whether inhibition of Src signalling affects *scribble* competition. We chose to block Src signalling with the PP2 inhibitor which has been previously shown to prevent the extrusion of Src-transformed cells from MDCK epithelia (Hogan *et al*. 2009). In the long time-frame of competition experiments, the published doses of 20 µM and 50 µM were toxic both to normal and mutant cells. However, when we reduced PP2 concentration to 4 µM, we no longer observed cell-autonomous death. Moreover, 4 µM protected *scrib*$^{KD+TET}$ cells from elimination by competition (Fig.7.6), suggesting that Src signalling is required for the elimination of the losers.

Encouraged by the effect that PP2 had on *scribble* competition, I decided to test the role of Src in the p53 MDCK competition model. First, having observed that PP2 delayed the expansion of wild-type clones in the *scribble* competition assay (compare unlabelled clones in Fig.7.6a and b), I decided to further titrate the inhibitor. In this way, I was hoping to identify a

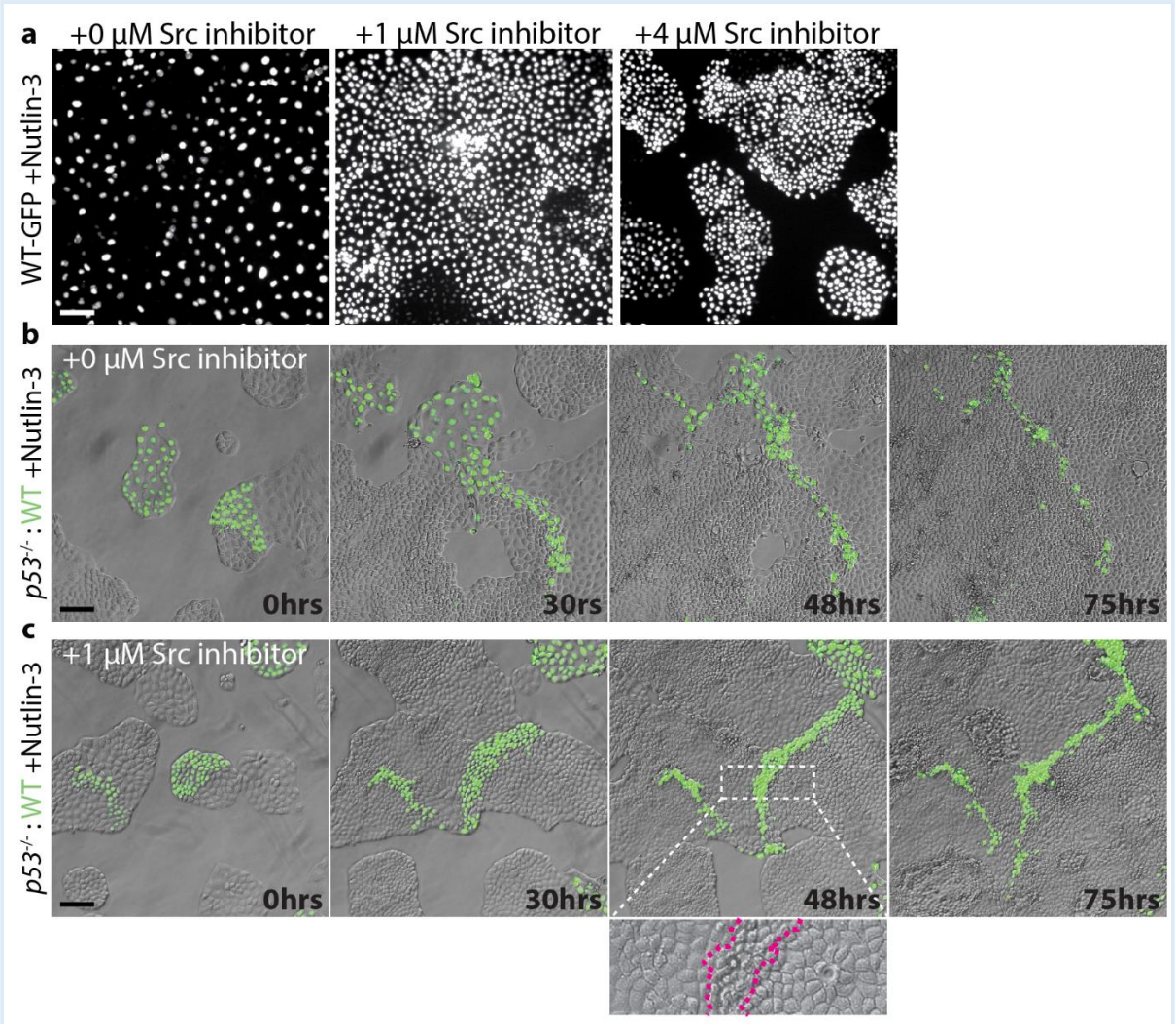


Figure 7.7. Inhibition of Src reduces sensitivity to crowding and blocks out-competition of p53-high cells.

(**a**) Dose-response assay testing the effect of Src inhibition (PP2) on WT cells treated with Nutlin-3. Presents stills from time-lapse, taken 84 h into treatment, and showing GFP-labelled nuclei. (**b**,**c**) Stills from time-lapse movies of GFP-labelled WT and unlabelled *p53*$^{-/-}$ cells co-cultured with Nulin-3 and without (**b**) or with (**c**) Src inhibitor (PP2). (**c**) Inhibition of Src blocked out-competition of GFP-labelled WT clones, but did not prevent their compaction; pink dotted lines surround GFP-labelled clone. Scale bars = 100 μm.

concentration of PP2 that does not significantly affect the winner cell population, but that has an effect on competition. Strikingly, when testing a range of PP2 concentrations on homotypic cell cultures, I found that 1 μM PP2 strongly rescued the density of p53-high cells (Fig.7.7a), suggesting that activation of Src signalling may trigger the hypersensitivity to cell density that is a characteristic of mechanical losers. I tested this hypothesis by adding 1 μM PP2 to p53 competition assays, and observed a marked

reduction in the elimination of p53-high cells (Fig.7.7b-c). Moreover, cell density within p53-high clones was higher than in the surrounding p53-low cells (Fig.7.7c, lower panel), arguing that PP2 did not prevent active compaction of p53-high cells, but that it protected them from out-competition by reducing their sensitivity to crowding.

7.3. **Discussion**

7.3.1. Integrin signalling as a candidate factor in mechanical competition

Activation of integrin signalling with Mn^{2+} did not prevent compaction of loser *scrib*$^{KD+TET}$ clones during competition, but it did inhibit their elimination. The accumulated evidence suggests that Mn^{2+} blocked competition by reduced the hypersensitivity to crowding displayed by *scrib*$^{KD+TET}$ cells. Possible explanations for this phenomenon include: (1) The anoikis theory, where compaction reduced the number of FAs and thereby triggers death via deprivation of survival signalling. (2) A variation of 1, where compaction disrupts FAs, but death is triggered indirectly by the translocation of FA-associated proteins to other signalling complexes. (3) FAs are not involved in mechanical competition, and ectopic activation of integrins protected *scrib*$^{KD+TET}$ cells from out-competition, by independently boosting their fitness. Further experiments will be required to distinguish address these possibilities. (1) The anoikis theory may be directly tested by culturing single cells on micro-patterns, where the cells could only adhere to adhesive spots of a defined size. Were the anoikis theory to be correct, the cells would not be able to survive on adhesive areas of the size available to compressed losers during competition. (2) I observed a translocation of two components of FAs to cell-cell junctions upon compaction. Both vinculin and P-Src can associate with adherens junctions (Woodcock *et al*. 2009; Fujita *et al*. 2002), and hence their translocation could be of significance to competition. To test whether the translocation of FA proteins to cell-cell

membrane is required in competition, a candidate-based approach could be used, where individual proteins are miss-localised by genetic means. It would be interesting to apply this approach to further investigate the role of Src in competition. (3) Around 24 integrins are formed by heterodimerisation of 18 α and 8 β subunits, only some of which were detected in MDCKs (Teräväinen *et al*. 2013). The requirement of integrin signalling for competition could be confirmed by first identifying those integrins that are affected by compression, and then by individually, genetically or chemically, modulating their function.

Preliminary experiments did not confirm that integrin signalling is required for p53 competition. Were this result to be confirmed, it could suggest either that *scribble* knock-down elevates p53 by modifying integrin signalling, or that integrin signalling plays a p53-independent function in competition.

7.3.2. Src is the first identified effector of p53 mechanical competition

Previously, we have identified a number of molecules involved in *scribble* mechanical competition. Such molecules could either: (1) Trigger competition by mildly upregulating p53 in *scrib*$^{KD+TET}$ cells. (2) Signal downstream of p53 to establish a loser phenotype. (3) Further boost p53 in response to compaction. (4) Trigger cell death in compacted cells downstream of p53. (5) Contribute to competition independently of p53. (6) Act through a combination of any of the above. By comparing the role of a candidate protein in *scribble* and p53 competition, we can exclude possibilities 1 and 5. In this way, I established Src as the first protein conclusively proved to affect mechanical competition in a p53-related manner; a result which should be further confirmed by genetic means. However, distinguishing between possibilities 2, 3 and 4 poses further challenges. The fact that p53-high clones reach higher densities than the surrounding culture even in presence of PP2 suggests that Src is not required for p53-high cells to be recognised as losers and actively

compacted by winners, arguing against possibility 2. Instead, the accumulated data suggests that PP2 blocks competition by reducing the sensitivity to compaction of p53-high cells, pointing towards possibilities 3 and 4.

Further studies will be required to test whether Src acts upstream (3) or downstream (4) of p53 in response to compaction. The first possibility seems more likely considering that Src localises to FAs and cell-cell junctions, both of which are known to be directly involved in response to mechanical stimuli (DuFort *et al*. 2011). A comparison of the effect of compression on p53 levels in presence and in absence of PP2 might confirm that Src activates p53 in response to compaction. Were this the case, the next question would be whether the effect of Src is mediated via ROCK and p38. Multiple lines of evidence place Src in indirect control of ROCK, which it can either activate or inhibit depending on context (Huveneers & Danen 2009); a possibility that should be further tested.

Chapter 8. DISCONTINUITY IN P53 SIGNALLING TRIGGERS DIRECTIONAL CELL MIGRATION

8.1. Introduction

During mechanical competition, scribble-deficient clones are actively compacted by wild-type cells. This compaction is a result of highly directional, collective cell migration that commences upon contact between *scribble*-deficient and wild-type clones, and where the *scribble*-deficient clones always migrate in front of the wild-types (Fig.1.4). Interestingly, directional cell migration also compacts loser clones in the MDCK p53 competition model, with loser cells (p53-high) again migrating in the front and winner clones (p53-low) at the back (Laura Wagstaff, unpublished data). This suggests that discontinuity in p53 signalling is sufficient to trigger directional cell migration.

The collective migration observed in mechanical competition models bears resemblance to another phenomenon observed in MDCK culture, where cells found at the migrating front and back are also morphologically-different. When confluent MDCK cultures are presented with an empty surface, they engage in collective migration to fill the vacant area. However, the migrating front is uneven, with characteristic multicellular finger-liked protrusions that contain a single leader cell at the tip in place of a fingernail (Poujade *et al*. 2007). These specialized leader cells differ from the followers. They display enlarged cell size, a lamellipodium, large focal adhesions, and accumulation of active Rac, integrin β1 and PI3K at their leading edge (Poujade *et al*. 2007; Rausch *et al*. 2013; Reffay *et al*. 2014; Yamaguchi *et al*. 2015).

Finger-like protrusions with a single leader cell are not unique to MDCKs and are found e.g. in IAR-2 rat liver cells (Omelchenko *et al*. 2003) and in

human breast adenocarcinoma line MCF-7 (Riahi *et al*. 2015). Leader-follower organisation is not restricted to epithelia, and has been for instance reported during migration of neural trunk cells (Richardson *et al*. 2016). Directional cell migration with one or more leaders at the front has been implicated in development, in healing of large wounds and in cancer (reviewed by Mayor & Etienne-Manneville 2016).

Several factors have been suggested to influence the formation of finger-like protrusions in epithelial cultures. These include geometric clues from the substrate that affect cytoskeletal tension (Rausch *et al*. 2013), RhoA signalling distribution, and continuity of an acto-myosin belt, which outlines the sides of the fingers and likely prevents formation of additional leaders (Reffay *et al*. 2014). Despite these findings, there has been little insight into why some cells, and not others, become leaders when confronted with an unoccupied surface. A recent study on MCF-7 cells addressed this question. The authors reported that a cell-free region reduces mechanical stress and increases DII4 expression, creating leaders. The frequency of leader cells was then controlled by Notch1–Dll4 lateral inhibition, where high levels of DII4 in leaders resulted in low levels of DII4 in the neighbouring cells (Riahi *et al*. 2015).

Despite these recent advances, the mechanisms that control leader-follower migration of epithelial cells are still not fully understood. Considering the apparent similarities between directional migration in mechanical competition and in the formation of finger-like protrusions, I hypothesized that common molecular mechanisms may govern both phenomena. In particular, I postulated that elevated p53 signalling may be a common characteristic of leader cells.

8.2. **Establishing a novel tissue culture model to study directional cell migration**

Leader cells found at tips of finger-like projections in epithelial cultures have a characteristic flattened morphology, with a large and active lammellipodium at the migrating front. Laura Wagstaff noticed that these leaders often, but not always, have more than one nucleus. This suggested the possibility that multinucleation might induce p53 activation and that p53 activation in turn might induce collective cell migration. This observation allowed me and Laura to develop a directional migration assay, where we could generate leader cells on demand, instead of having to rely on spontaneous appearance of finger-like projections, as had been the case in previous studies. We generated multinucleated cells by pre-treating GFP-labelled MDCKs with Blebbistatin, a Myosin-II inhibitor that blocks cytokinesis without stopping karyokinesis (Straight *et al*. 2003). I tested a range of Blebbistatin concentrations and incubation times, and selected a 16 hours incubation in 37.5 μM Blebbistatin as the optimal conditions to generate a population where most (>80%) cells have two or more nuclei, with the minimal effect on cell viability. The vast majority of the generated cells were binucleated, and the number of nuclei stayed stable after removing Blebbistatin; i.e. karyokinesis did not resume, and further cytokinesis was blocked. Having developed an efficient method to generate leaders, we then mixed the GFP-labelled multinucleated cells (putative leaders) with unlabelled wild-types (putative followers), to induce collective cell migration. The use of a nuclear GFP label allowed not only to distinguish between leaders and followers, but also to easily confirm the number of nuclei per cell. Notably, upon contact with multinucleated cells, the wild type clones engaged in directional migration (Fig.8.1a), confirming the validity of the approach.

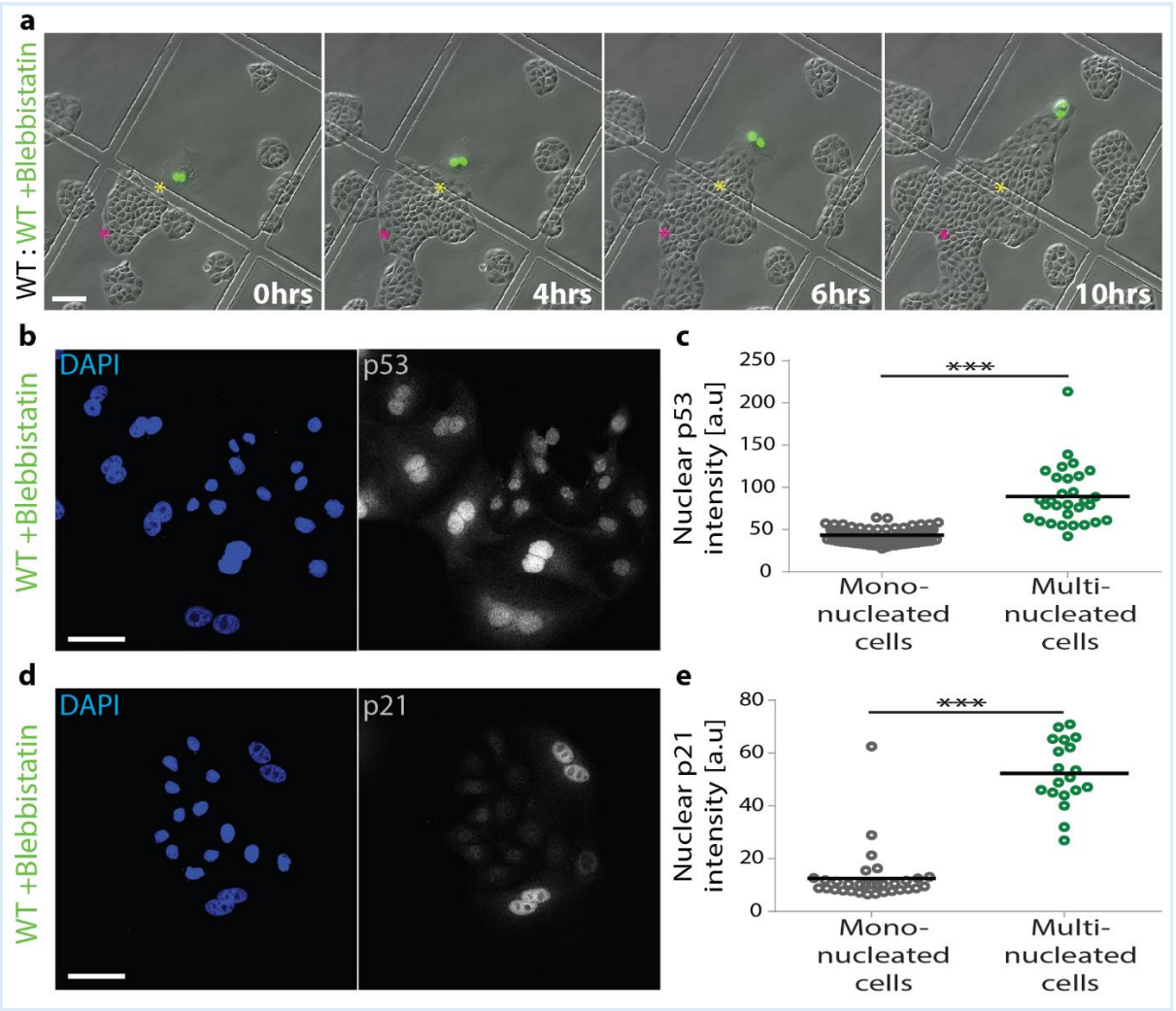


Figure 8.1. Multinucleated cells show elevated p53 signalling.

(**a**) Stills from time-lapse imaging. GFP-labelled multinucleated leaders (generated by pre-treatment with Blebbistatin) engaged in directional migration with unlabelled WT followers. Yellow star marks initial point of contact between a multinucleated cell and a WT clone; pink star shows opposite edge of the WT clone. Scale bar = 100 µm. (**b**,**d**) Representative confocal images of cultures pre-treated with Blebbistatin and then left to recover for 2 days, for the remaining mononucleated population to expand. (**b**) Staining against p53. (**c**) Single-cell nuclear p53 intensity from images as in **b**. (**d**) Staining against the p53 transcriptional target, p21. (**e**) Single-cell nuclear p21 intensity from images as in **d**. (**b**,**d**) Scale bars = 50 µm. Black bars = mean. ***; $p<0.0005$ by KS test.

8.3. **Multinucleated cells have elevated levels of p53 signalling**

The first step in testing whether p53 may be involved in the formation of finger-like protrusions, was to measure the levels of p53 signalling in multinucleated cells. For this purpose, I pre-treated MDCK cells with Blebbistatin, and then left the cultures for two days to recover, allowing the remaining population of mononucleated cells to expand. By comparing p53 levels between multi- and mononucleated cells that have been subjected to the same treatment, I attempted to exclude any effect of Blebbistatin on stress levels that was unrelated to the number of nuclei.

By immunofluorescent staining, I detected an approximately two fold increase in nuclear p53 levels in multinucleated cells, when compared to mononucleated cells (Fig.8.1b-c). While the levels of p53 signalling in multinucleated cells were heterogeneous, they were nearly always higher than the average for wild-type cells, suggesting that upregulation of p53 is a common response to multinucleation. To further confirm the activation of p53 signalling, I measured nuclear levels of p21, an established transcriptional target of p53 (Zilfou & Lowe 2009). p21 is a cyclin-dependent kinase inhibitor, and the most strongly up-regulated p53 target in *scrib*$^{KD+TET}$ cells, as detected by transcriptional profiling (Table.3.1). Similarly, p21 levels were robustly (about four fold) elevated in multinucleated cells when compared to wild-type cells (Fig.8.1d-e), arguing for an increase in transcriptional activity of p53, and suggesting similarities between *scrib*$^{KD+TET}$ and multinucleated cells.

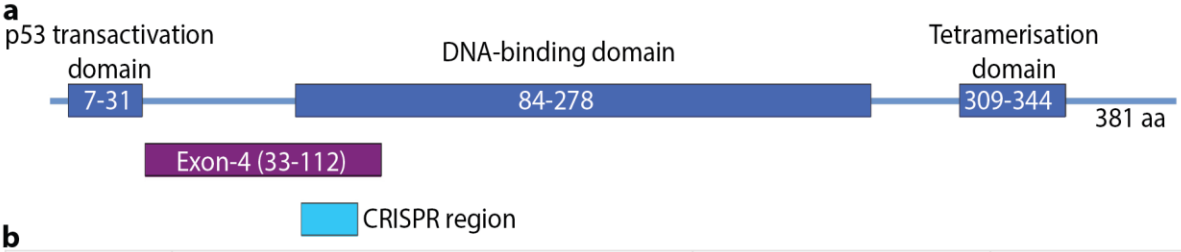
8.4. **Generating and characterising p53-deficient clones**

Having established that p53 signalling is elevated in multinucleated leader cells, I then asked whether it is required for these cells to engage in directional migration. One way to directly address this question would be to remove p53 from the leaders, and test whether this prevents directional

migration. To attempt this approach, I first had to obtain p53-deficient cells, from which I could then generate leaders. Others from our group had recently generated a p53-deficient population by targeting the N-terminus of the DNA-binding domain (DBD) of p53 by CRISPR (Fig.8.2a). I decided to use this pool to isolate and characterise several clonal populations. In this way, I obtained a p53-null clone (*p53*$^{-/-}$) and three clones that express p53, but with deletions (*p53*$^{DBD\text{-}1}$), insertions (*p53*$^{DBD\text{-}3}$), or both (*p53*$^{DBD\text{-}2}$), in the targeted region (Fig.8.2, Supplementary Data-4). I only detected a single allele of Trp53 in clone *p53*$^{DBD\text{-}1}$, suggesting either that only one copy of this gene in present in these cells, or that the same mutation occurred in multiple loci (Fig.8.2b,c). In contrast, I detected two distinct mutations in clone *p53*$^{-/-}$ and *p53*$^{DBD\text{-}3}$ at approximately equal frequency (Fig.8.2b,c), suggesting that these clones carry two alleles of Trp53. In clone *p53*$^{DBD\text{-}2}$ one of the detected alleles was underrepresented, which may argue that the population was contaminate with another mutant clone (Fig.8.2b,c). To verify these interpretations, it will be necessary to determine the number of p53 loci in each clone. While MDCKs are nearly diploid, a cell-to-cell variation in the number of chromosomes has been reported (Cassio 2013).

We used two additional approaches to test p53 status in each clone. Paola Marco-Casanova from our group confirmed the absence of p53 expression in *p53*$^{-/-}$ and the predicted shift in protein size in clone *p53*$^{DBD\text{-}3}$ by Western blot. As expected, p53 was expressed at unchanged levels in clone *p53*$^{DBD\text{-}1}$; the predicted one amino-acid change in protein size was below detection range. Independently, I tested whether the transcriptional function of p53 has been disrupted, by staining against a transcriptional target of p53 (p21). p21 was no longer activated in multinucleated cells generated from clone *p53*$^{-/-}$, and only weakly and sporadically upregulated elevated for *p53*^{DBD} clones (Fig.8.3), indicating that transcriptional activity has been completely lost in the p53-null clones, and mostly lost in the clones carrying mutations in the DBD domain.

a



b

Clone	Mutation (location in Exon-4)	Prevalence	Frameshift
p53^{DBD-1}	Δ203-211 (9 bp)	100%	No
p53^{DBD-2}	Δ194-205 (12 bp)	89%	No
	173::174 (7 bp) & 192::193 (12 bp)	11%	Yes
p53^{DBD-3}	188::189 (42 bp)	56%	No
	188::189 (102 bp)	44%	No
p53$^{-/-}$	Δ168-201 (34 bp)	56%	Yes
	210::211 (7 bp)	44%	Yes

c

Clone	Translation of Exon-4; starting at amino acid 40
WT	PGPAPSWPLSSSVPSPKTYPGTYGFRLGFLHSGTAKSVTWT
p53^{DBD-1}	PGPAPSWPLSSSVPSPKTYPGTYGFRLG---SGTAKSVTWT
p53^{DBD-2}	PGPAPSWPLSSSVPSPKTYPGTYGFL----HSGTAKSVTWT
	PGPAPSWPLSSSVPSPKT*
p53^{DBD-3}	PGPAPSWPLSSSVPSPKTYPGTYGFRLGFLHWHLWVPFGVPTLAPMGSVWGSCIGTYGFRLGFLHSGTAKSVTWT
	PGPAPSWPLSSSVPSPKTYPGTYGFRLGFLQTYPGTYGFRLGFLHSGTAKSVTWT
p53$^{-/-}$	PGPAPSWPLSSSVPSPNSCIPGQPSLLLG
	PGPAPSWPLSSSVPSPKTYPGTYGFRLGFLHPAFRDSQVCYLD

Figure 8.2. p53-deficient MDCK clones carry mutations within Exon-4 of TP53 gene. (**a**) Schematic structure of dog p53, based on Transcript ID ENSCAFP00000024579.3. CRISPR mutagenesis targeted Exon-4, at the beginning of DNA-binding domain (DBD). (**b**,**c**) TP53 region targeted by CRISPR mutagenesis was sequenced multiple times (see Methods section) for each mutant clone. (**b**) Detected mutations within Exon-4 and (**c**) corresponding amino acid sequences.

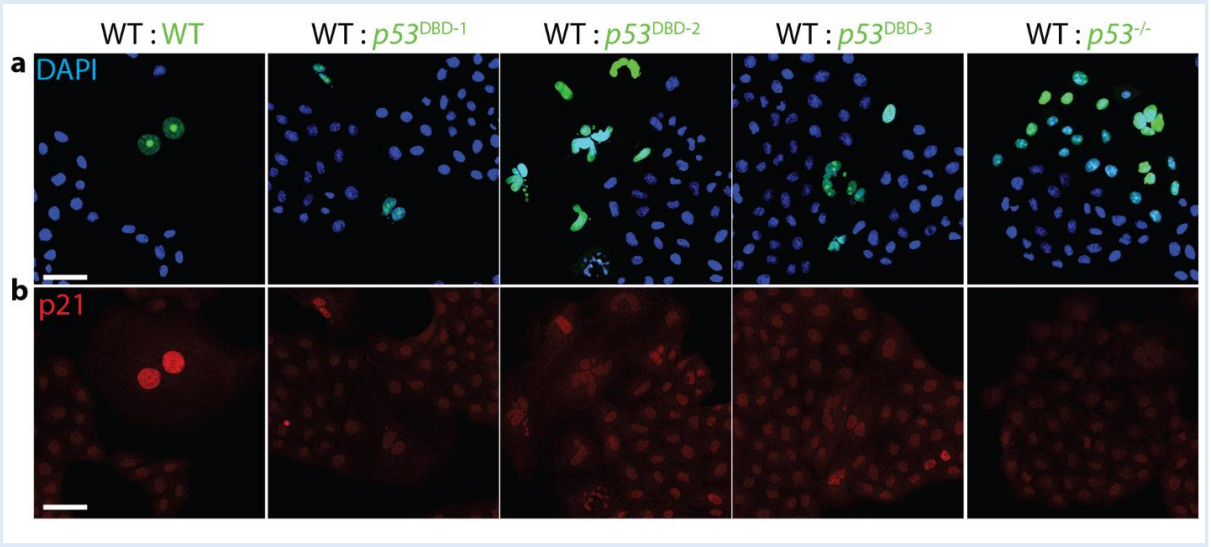


Figure 8.3. All p53-mutant clones lost transcriptional activity of p53.
(**a**) Confocal images from co-cultures of unlabelled WT cells (counterstained with DAPI), and GFP-labelled cells pre-treated with Blebbistatin. (**b**) Corresponding staining against the p53-transcriptional-target, p21. Scale bars = 100 µm.

8.5. **A sub-population of multinucleated cells requires p53 for directional migration**

Having obtained a p53-null clone and three clones with mutations in the DBD domain, I proceeded to test whether multinucleated cells require p53 signalling to become leaders. For this purpose I generated multinucleated cells by pre-treating GFP-labelled wild-types (control) or p53-mutants with Blebbistatin. I then mixed these multinucleated cells with unlabelled wild-type cells and followed their fate by live imaging (Fig.8.4). As expected, nearly all control multinucleated cells were recognised as leaders, and induced vigorous collective migration upon contact with mononucleated cells (Fig.8.4c). In contrast, only approximately half of the imaged p53-mutant clones engaged in directional mutation (Fig.8.4). The proportion of p53-mutant cells recognised as leaders was comparable between *p53*$^{-/-}$ cells and all *p53*^{DBD} mutants, enforcing the obtained result. Together, this data suggests that elevated p53 is a trigger of collective cell migration, but also

points to the existence of parallel, p53-independent mechanisms that lead to the formation of finger-like projections in MDCK cell culture.

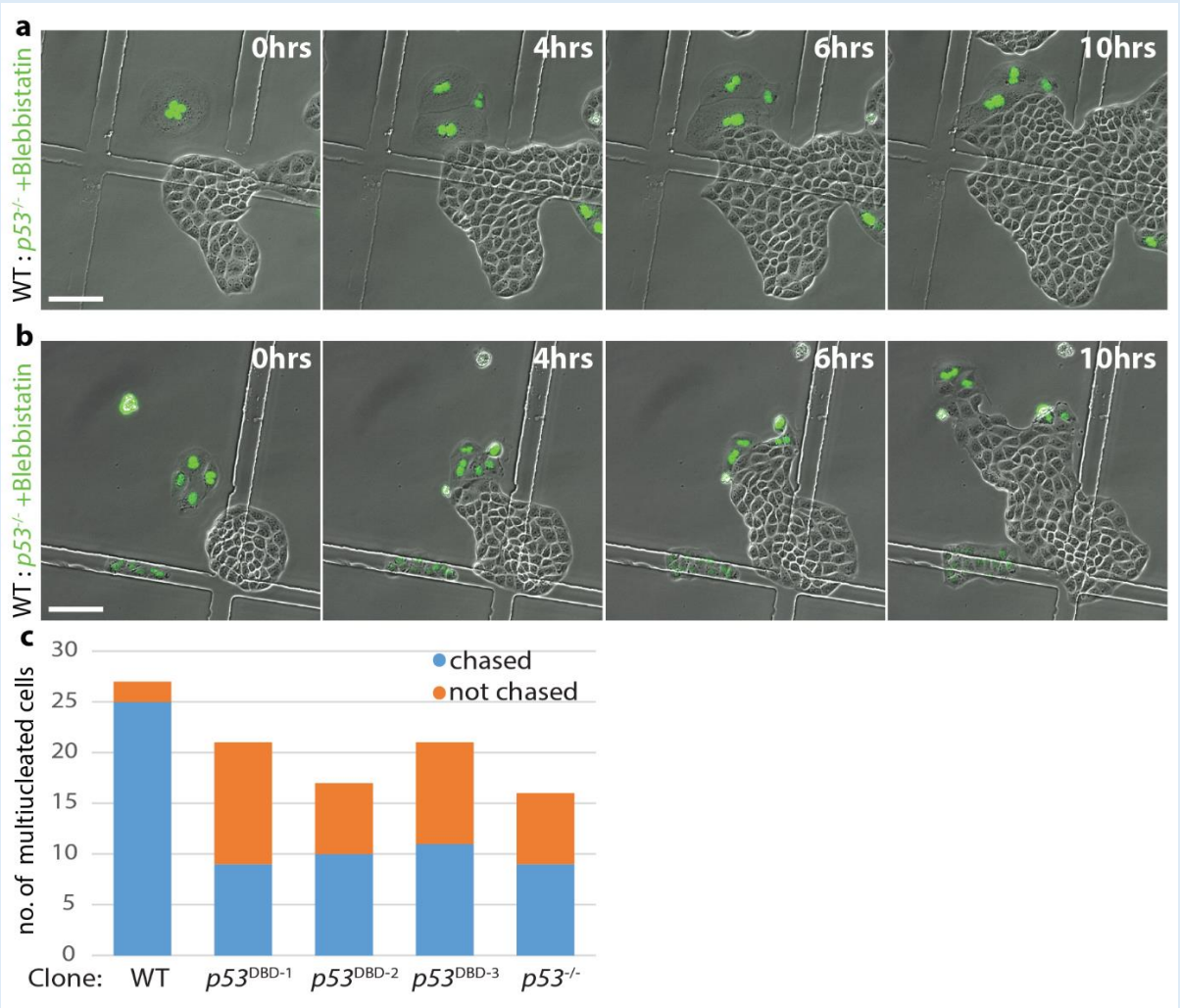


Figure 8.4. Activation of p53 is involved in leader status of multinucleated cells. (**a**-**b**) Stills from time-lapse imaging of GFP-labelled multinucleated prospective leaders, co-cultured with unlabelled WT cells. Examples of *p53*$^{-/-}$ multinucleated cells that engage (**b**) or do not engage (**a**) in collective migration with WT cells. Scale bar = 50 μm. (**c**) Fraction of p53-deficient multinucleated clones that become leaders, from images as in **a** and **b**.

8.6. **Discussion**

8.6.1. A novel model to study leader-follower migration of epithelial cells

Based on the observation that leader cells in MDCK culture are often binucleated, we established a new model to study directional cell migration, in which leaders can be created on demand. This presents a huge advantage over the established practice of studying spontaneously occurring leaders, as it allows us to separately manipulate leaders or followers, by genetic or chemical means, before they are confronted with each other. This approach has already proven useful in characterising the role of p53 signalling in leader cell migration, and could be further exploited for candidate-based studies and for large scale screens. Since not all MDCK leaders are multinucleated, any potential findings, including the requirement for p53 signalling, will have to be further tested on spontaneously occurring leaders, to establish whether they apply to all leaders, or are specific to multinucleated cells.

8.6.2. P53 is involved in leader-follower migration

I have shown that p53 signalling is upregulated in binucleated cells and that it is required for some, but not all, of these cells to become leaders, suggesting that parallel, p53-dependent and independent mechanisms, trigger leader-follower migration. To the best of my knowledge, this is the first study that implicates p53 signalling in leader-follower migration. Elevated p53 signalling has been previously reported to activate migration of primary adult human pulmonary fibroblasts (Wang *et al*. 2015), however, no connection has been made to collective cell migration. A migratory phenotype has been more commonly associated with p53-deficiency or gain-in-function mutations, but again not in the context of leader-follower migration (Roger *et al*. 2006; Muller *et al*. 2011).

Interestingly, the effect on migration was indistinguishable between p53-null cells all three clones that carry mutations in the DNA-binding domain. This suggests that p53 affects the leader status by regulating transcription. This hypothesis could be confirmed by demonstrating that the *p53*^{DBD} clones retained their non-transcriptional functions, such as the ability to trigger the intrinsic apoptosis pathway (Vaseva & Moll 2009).

8.6.3. Directional cell migration in competition and in the formation of finger-like protrusions

Mechanical losers and leader cells at the tips of finger-like projections in MDCK culture share several characteristics, such as a flattened morphology, large focal adhesions, the ability to trigger migration upon contact with wild-type cells and their position in front of wild-type cells during migration. These parallels suggest that both types of migration may be in fact different models of the same phenomenon. Based on this hypothesis, I proposed that p53, which is involved in directional migration during competition, may also play a role in the formation of finger-like projections; a prediction that proved correct. In continuing to explore the parallels between both models of migration, one could advance the understanding of mechanical competition by testing the role of known effectors of leader-follower migration, such as Rho-A, Rac, integrin β1 and PI3K, in mechanical losers.

Chapter 9. DISCUSSION

9.1. MTEC cultures provide a novel model to study competition in adult mammalian epithelia

Despite the recent interest in mammalian competition, most research on adult epithelia has been carried out in MDCK cultures, creating a demand for alternative models. Here, I developed a new competition model that presents several unique advantages, in addition to allowing the possibility to validate results obtained in MDCK cultures. The primary MTEC cultures provide a pseudo-*in-vivo* system, which consists of Basal stem cells and two types of differentiated columnar cells. Therefore, while relatively simple in structure, the tracheal epithelium allows to study the relative contribution of stem and differentiated cells in cell competition; a topic that has been scarcely explored. The MTEC cultures are suitable for prolonged experiments, often required to study mammalian competition, and are convenient for live-imaging. Moreover, any results obtained *in vitro* could be readily confirmed *in vivo*, as tracheal epithelia are easily accessible for treatments. Following on the success of establishing a p53 competition assay, other types of competition could be modelled in the MTEC system.

9.2. Mechanical competition eliminates loser cells by compaction

The density of *scribble*-deficient MDCK cells in homotypic cultures is much lower than that of wild-type cells, but it drastically increases during competition. This led us to hypothesize that the out-competition of *scribble*-deficient cells occurs via mechanical compaction, and not by biochemical signalling. Here, by artificially increasing cell density in homotypic cultures, I conclusively demonstrated that enforced compaction is sufficient to kill *scribble*-deficient losers in a complete absence of a winner cell population.

We termed the phenomenon in which loser cells are eliminated by mechanical means, mechanical cell competition.

Consistent with previous reports that apoptosis is the main mode of elimination of *scribble*-deficient cells from competing co-cultures (Norman *et al*. 2012), I demonstrated that compaction is sufficient to trigger robust apoptosis of the mutant cells. Moreover, *scribble*-deficient cells died even at densities lower than the homeostatic density of wild-type cells, emphasizing their extreme hypersensitivity to crowding. Furthermore, I provided further insight into the role of apical extrusion in *scribble* competition. Although present in competing cultures, extrusion was not required for out-competition of *scribble*-deficient cells (Norman *et al*. 2012). Indeed, inhibition of signalling cascades reportedly involved in the elimination of apoptotic (S1P2 inhibitor; Gu *et al*. 2011), or supernumerary MDCK cells (S1P2 and Piezo1 inhibitors; Eisenhoffer *et al*. 2012) did not affect competition. This finding is interesting as it demonstrates that the response of wild-type and *scribble*-deficient cells to enforced compaction is not only quantitatively, but also qualitatively different, with live-extrusion as the predominant response of normal, and death as the response of mutant cells.

A striking characteristic of mechanical losers is their reduced homeostatic density and an accompanying flattened morphology. This poses the question of whether a flattened morphology, and hypersensitivity to compaction, are necessary and sufficient to mark cell as a mechanical losers. Further studies will be required to test if all abnormally spread cells are recognised as mechanical losers. As for whether hypersensitivity to compaction is required for mechanical competition, according to a recent report, normal cells can be eliminated by mechanical super-competitors (Levayer *et al*. 2016), arguing that a relative difference in the sensitivity to compaction is sufficient to trigger competition.

9.3. **Elevation of p53 is the hallmark of mechanical loser phenotype**

I set out to characterise the molecular signature of prospective loser cells by transcriptional analysis. First, I compared transcriptomes of normal and *scribble*-deficient cells, to identify transcripts affected by *scribble* RNAi. I then contrasted transcriptomes of *scribble*-deficient cells and *scribble*-deficient cells resistant to competition, to enrich for those genes affected by *scribble* silencing that correlate with the loser status. This approach proved vastly successful, as it allowed me to identify p53 as the key determinant of mechanical loser status. In depth analysis of other candidate genes and pathways identified in these comparisons may yield further valuable insights into the loser cell status.

Further studies on the role of p53 in competition revealed that sub-lethal elevation of p53 is sufficient to turn otherwise normal cells into mechanical losers. I contributed to the discovery that p53-high MDCK cells are eliminated by mechanical means, and demonstrated that mechanical p53 competition is not an artefact of MDCK culture, but that it is conserved in tracheal epithelia. In particular, I observed that p53-high 16Hbe tracheal epithelial cells were hypersensitive to compaction, when compared to p53-normal cells, and that, in primary MTEC cultures, p53-high clones had lower homeostatic cell density than wild-type clones, but were compacted during competition. It remains to be seen whether p53 mechanical competition is prevalent in mammalian epithelia or if it is tissue-specific. p53 is ubiquitously activated in response to stress, suggesting that all tissues may be capable of p53-driven competition. However, the link between p53 and hypersensitivity to density may not be as universal, and it should be further investigated which epithelial cells flatten upon activation of p53. Moreover, the mechanisms by which p53 causes flattening remain unclear. Compared to the vast literature of p53-mutants, and on the role of p53 in apoptosis, relatively little attention has been paid to the effects of mild activation of

p53 in general, and to its effect on cell shape in particular (Araki *et al*. 2015).

9.4. **The many roles of p53 in the elimination of stressed cells**

The elimination of p53-high cells in mammalian epithelia follows three distinct stages: (1) Acquisition of a loser cell status. As discussed above, sub-lethal activation of p53 signalling is sufficient to render cells into mechanical losers. The cells undergo morphological changes (flattening) and become hypersensitive to density. (2) Upon contact with winners, losers are actively compacted to abnormally high cell densities. Laura Wagstaff reported compaction of p53-clones in competing MDCK cultures. I then observed the same phenomenon during MTEC competition. Although the initial activation of p53 is sufficient to trigger both hypersensitivity to density and active compaction, it remains to be seen whether these two phenomena are linked, or whether they are independent results of the activation of p53. (3) Enforced compaction triggers apoptosis. Laura Wagstaff reported that p53 is further boosted in loser clones upon compaction by winners. We propose that this additional activation of p53 tips the balance from non-apoptotic to pro-apoptotic p53 signalling. I directly demonstrated that p53 is required for compaction-induced death of MDCK cells by analysing how homotypic cultures of *scribble*-deficient cells respond to overcrowding in the absence of p53 signalling (p53 knock-out). This confirms that p53 is involved at all stages of the elimination of p53-high cells. However, at present, we have only indirect evidence that compaction-induced boosting of p53 is required for compaction-induced death. Instead, it is possible that the initial, sub-lethal, activation of p53 confers a hypersensitivity to compaction that leads to compression-induced death in a p53-independent manner. Further experiments will be required to address this possibility.

We demonstrated that p53-high cells, in MDCK and MTEC cultures, are eliminated by mechanical means. This poses the question of whether all models of mechanical competition are p53-dependent. A first step to address this question could be to investigate the role of p53 in the recently-reported mechanical super-competition models, where normal cells were eliminated by Ras-transformed cells in *Drosophila* pupal midline (Levayer *et al*. 2016). Conversely, one may ask if p53 competition always occurs by mechanical means. To consider this question, one may investigate the established models of p53 competition. Several previous studies addressed the role of p53 signalling in cell competition and reported contradictory results. In *Drosophila*, activation of p53 has been linked either to the winner status, or had no apparent effect on competition. de la Cova and colleagues (2014) reported that elevated p53 signalling boosted fitness of Myc-overexpressing cells, and was required for them to act as super-competitors. In contrast, p53 status had no effect on *Minute* competition (Kale *et al*. 2015). Similarly, out-competition of *scribble*-deficient cells was not rescued by inhibition of p53 in wing imaginal discs. These results argue that the role of p53 in competition is not conserved between *Drosophila* and mammals. Conversely, the only previous study on p53 competition in a mammalian tissue supports our observations. Bondar and Medzhitov (2010) reported that p53-high cells out-competed p53-low cells in the bone marrow of adult mice, suggesting that p53-high cells may be universal losers in mammals. In light of our results, it would be interesting to test whether p53 competition in the bone marrow occurs via mechanical means. Furthermore, this model of competition provides a rare example where loser cells are eliminated by senescence rather than apoptosis. This poses the question of whether death is the only possible outcome of mechanical competition.

9.5. **Compaction activates p53 via ROCK and p38**

Having established that compaction kills mechanical losers, and that this requires p53 signalling, I decided to further investigate the molecular

mechanisms of this phenomenon. All experiments were conducted solely in the *scribble* MDCK competition model and may or may not have a conserved role in other p53-related competition models or culture systems. Based on data generated by myself and others, we propose that in response to compaction ROCK activates p38 leading to elevation of p53 and to cell death. The gathered evidence includes: (1) Compaction activates ROCK. I confirmed by immunofluorescence, showing the upregulation of three targets of ROCK (P-Myosin II, P-MYPT1 and fibrous actin) in compacted loser clones. I further demonstrated that ROCK signalling is required for *scribble*-deficient cells to be eliminated by competition and by enforced mechanical compaction in absence of a winner population. Furthermore, *scribble*-deficient clones were actively compacted by wild-type cells even in the presence of a ROCK inhibitor, suggesting that the mutants were still recognised as losers, and that ROCK signals downstream of compaction. (2) ROCK activates p38 in response to compaction. I demonstrated that compaction-induced activation of p38 is partially blocked in presence of a ROCK inhibitor, suggesting that p38 signals downstream of ROCK in response to overcrowding. (3) Activation of p38 is required for elimination of *scribble*-deficient cells in response to compaction. Others demonstrated that p38 signalling is required for out-competition of *scribble*-deficient cells (Norman *et al*. 2012) and that p38 is activated in response to compaction (Wagstaff *et al*. 2016). I clarified the role of p38 in mechanical competition by directly demonstrating that p38 signalling is required for enforced compaction to trigger death of *scribble*-deficient cells. (4) p38 signals upstream of p53 in response to compaction. Others demonstrated that inhibition of p38 blocks the elevation of p53 in response to compaction (Wagstaff *et al*. 2016). (5) p53 signalling is required for compaction-induced death. As previously discussed.

This is not the first instance when ROCK has been implicated in cell competition in MDCK culture. However, no previous reports describe activation of ROCK in the loser cells. Instead, during Ras and Src

competition, the activation of ROCK has been detected in the surrounding winners (Kajita *et al*. 2014). It is likely that, since ROCK signalling in the losers is involved in out-competition by apoptosis, and Ras- and Src-transformed cells are eliminated by live-extrusion, such up-regulation is not required for Ras and Src competition. Future studies on the involvement of ROCK in mechanical competition should clarify how compaction activates ROCK, how ROCK activates p38, and whether it can activate p53 independently of p38 signalling.

9.6. **Integrin signalling and Src in mechanical competition**

When further investigating how overcrowding triggers death of mechanical losers, I noticed that compaction drastically reduces the cell-substrate adhesion area. I hypothesized that this reduction disrupts FAs, thereby killing mechanical losers by deprivation of integrin survival signalling. To test this hypothesis, I fist carried out immunofluorescent staining against components of FAs (Vinculin and Src). While spread *scribble*-deficient cells contained large FAs located at the ends of stress fibres, in compacted cells these molecules translocated to cell-cell junctions, suggesting that either the disruption of FAs, or the alterations in cell-cell junctions, may play a role in the elimination of mechanical losers. I then decided to modulate integrin signalling to test its effect on *scribble*-deficient cells. Mild activation of integrin signalling with Mn^{2+} drastically increased the homeostatic density of mutant cells and reduced their death upon enforced compaction, suggesting that integrin signalling reduces hypersensitivity to density. Mn^{2+} treatment of competing co-cultures did not affect active compaction of *scribble*-deficient cells, but partially blocked their elimination. These results have not yet been successfully reproduced in the p53 competition model. Furthermore, additional experiments will have to be carried to determine whether activation of integrin signalling protects *scribble*-deficient cells by: (1) Rescuing a deficit in integrin survival signalling sustained upon compaction. (2) Preventing translocation of FA components into cell-cell

junctions, where they may activate pro-apoptotic signalling. (3) It is possible that integrin signalling is not normally be involved in *scribble*-competition, but that Mn^{2+} treatment protected the mutant cells by artificially boosting their fitness.

Encouraged by the likely involvement of integrin signalling in mechanical competition, I then investigated the role of a FA-associated kinase, Src, in mechanical competition. Src is of particular interest, as it has been previously implicated in competition in MDCK culture (Kajita *et al*. 2010). Chemical inhibition of Src in *scribble* and p53 MDCK competition models did not prevent active compaction, but efficiently blocked out-competition. Moreover, mild down-regulation of Src in p53-high monocultures drastically increased the homeostatic cell density, suggesting that Src signalling causes hypersensitivity to compaction. An enforced compaction experiment carried in absence of a winner cell population will be required to test whether activation of Src triggers apoptosis in response to compaction. Together, this data suggests that reduction in Src signalling protects mechanical losers by reducing their hypersensitivity to compaction. Further studies will be required to resolve the apparent contradictions between these and previous results. (1) Kajita and colleagues (2010) reported that cells with continuously-active Src signalling were eliminated by live extrusion and not by apoptosis (Kajita *et al*. 2010). If Src signalling confers hypersensitivity to compaction, then why are the transformed cells not killed when surrounded by wild-type cells? (2) Since Src is involved in FA survival signalling (Vachon 2011), and activation of integrin signalling protects mechanical losers, then why does inhibition, and not activation of Src, block mechanical competition? Src is known to perform various functions depending on what cellular compartment it localises to. Considering that Src localises predominantly to FAs and the nuclei in un-compressed loser cells, to nuclei and cell-cell junctions upon mild compression, and away from the nuclei in highly compressed cells, a better understanding on how these translocations affect competition may shed light on the above questions.

Similarly, a better understanding on how Src signalling is linked to ROCK, p38, and especially p53, may help to unravel its function in mechanical competition.

9.7. **Why does mechanical p53 competition matter?**

The morphology and function of epithelial cells are tightly linked. Alterations in cell shape are likely to negatively affect their function. Mechanical competition may provide means for removing such sub-optimal cells. The primary role of p53 is to coordinate responses to stress. Over a certain threshold, activation of p53 triggers cell-autonomous elimination of damaged cells. I propose that p53 mechanical competition provides a mechanism by which sub-optimal cells may be eliminated, but only when fitter cells are available to replace them. In this way, moderately damaged cells may temporarily serve as place-holders, helping to preserve the integrity of an epithelium until an undamaged cell population had a chance to expand. Moreover, since p53-high cells are actively compacted by normal cells, the present of a p53 loser might provide a "wake up" call to the surrounding cells, promoting their migration and expansion, and facilitating rapid repair of a damaged tissue.

p53 is the most commonly mutated protein in cancer, suggesting that p53 competition may, in addition to its homeostatic functions, play a major role in tumour progression. p53 competition could provide means by which p53-deficient cells propagate in an epithelium, thereby enlarging the mutant cell population and increasing the chance of accumulation of further mutations. It would be interesting to test whether cells from different developmental lineages engage in p53 competition, and thus whether p53-competition might facilitate metastasis to different tissues. An interesting observation supported both by our experiment in MDCK and MTEC cultures and by earlier studies in the bone marrow (Bondar & Medzhitov 2010), is that p53 competition required an external activator of p53 to create a discontinuity in the p53 signalling levels. It would be therefore interesting to test whether

tumour microenvironment is capable of activating p53 in normal cells, and thereby triggering p53 competition. Furthermore, the requirement for an activator of p53 signalling suggests that p53 competition could be particularly strong in those tissues that are subjected to external stress, such as UV irradiation of the skin, cigarette smoke in the lungs, or viral infections.

9.8. **P53 is involved in leader-follower migration**

Previous work from my group has shown that spontaneously binucleated cells in MDCK cells cultures are chased by wilt-type cells. My work has shown that the acquisition of a multinucleated state is sufficient to elevate both p53, and its transcriptional target p21 in MDCK cells. By establishing an experimental system where I could generate multinucleated cells on demand (based on Blebbistatin), and then confront them with wild-type followers. I discovered that approximately half of the p53-deficient multinucleated cells did not trigger leader-follower migration. This suggests that discontinuity of p53 signalling is an important, but not the only, mechanism that drives the formation of finger-like protrusions. Furthermore, I observed no difference in the effect on migration between p53-null cells and cells carrying mutations in the DNA-binding domain of TP53. This suggests that p53 may trigger directional migration by regulating transcription, as opposed to by its transcriptional-unrelated functions, a result that requires further verification.

Mechanical losers and multinucleated cells at the tips of finger-like projections share several characteristics, including a flattened morphology and elevated p53 levels. Since activation of p53 signalling is sufficient to bestow a loser cell phenotype, it would be interesting to test whether multinucleated cells and, more broadly, all leader cells, are also mechanical losers. One could hypothesize that stressed and potentially damaged cells at the site of injury flatten to cover a de-cellularized area and assure the integrity of the epithelium. At the same time these loser cells trigger

directional cell migration, which gradually covers the damage area with intact cells. When cell density increases sufficiently, and the healing nears completion, the p53-high cells are removed by mechanical competition. Since not all multinucleated cells required p53 signalling to become leaders, it would be also interesting to test whether these p53-independent leaders act as mechanical losers. In this way, one could address the question of whether p53 status and mechanical loser status are always linked.

p53-driven directional migration may prove a major and common mechanism of wound closure. Cells placed close to the site of injury are likely to be stressed, and hence to elevate p53. p53-driven directional migration does not have to be limited to the formation of finger-like protrusions, but could be a more universal phenomenon in wound healing. Interestingly, *in vitro* and *in vivo* injury to tracheal epithelia does not result in the formation of finger-like protrusion, but triggers rapid flattening of all cells adjacent to the site of injury (Fig.6.1, You *et al*. 2002; Paul *et al*. 2014). This flattened morphology resembles that of p53-high MDCK cells, and invites further investigation into the role p53 in healing of the trachea.

Chapter 10. METHODS

10.1. Tissue culture

10.1.1. Immortalised cell lines

Eph4 cells were kindly provided by Guido Posern (Martin Luther University Halle-Wittenberg, Germany) HEK293T cells by Steven Jackson (University of Cambridge, UK), and 16Hbe cells by Sandrine Etienne-Manneville (Institut Pasteur, France).

Wild-type MDCK, MDCK-pTR *E-cadherin* shRNA (*Ecad*^{KD}) and MDCK-pTR *scribble* shRNA (*scrib*^{KD}) cells were a kind gift from Yasuyuki Fujita (Hokkaido University, Japan). *scrib*^{KD} cell lines expressing a nuclear green fluorescent protein (GFP), and competition-resistant *scrib*^{KD} MDCK cells (*scrib*^{RES}) were generated by Laura Wagstaff from the Piddini group.

The Piddini group generated pools of *p53*$^{-/-}$ and *scrib*^{KD} *p53*$^{-/-}$ cells (Wagstaff *et al*. 2016), using Cas9 D10A CRISPR technology with the following sgRNAs:

p53_CRISPR#1_Fw: 5'-GGTGCCAGGGTAGGTCTTCG -3'

p53_CRISPR#2_Fw: 5'- GTTTGGGGTTCCTGCATTCC -3'

I then labelled the *p53*$^{-/-}$ pool with pGIPZ-NlsGFP-Puro by lentiviral infection followed by a 7-day selection in puromycin (0.65 µg ml^{-1}, Sigma). To isolate different *p53* mutants I then generated clonal populations by plating cells at 1 cell per well in 96-well plates and expanding the resulting clones.

10.1.2. Primary mouse tracheal epithelial cells

Primary mouse tracheal epithelial cells (MTECs) were obtained from 5-month-old animals from *Rosa*26R-Tomato (*Gt(ROSA)26Sor*$^{tm1(CAG-tdTomato*,-}$

EGFP)*Ees) and p53-null (*Trp53*^{tm1tyj}) strains, both of C57BL/6 background, using a protocol adapted from published methods (You *et al*. 2002). The mice were culled by terminal anaesthesia. Tracheas were dissected from the larynx to the bronchial main branches and collected in ice-cold DMEM:F12 (11330-32; Invitrogen) supplemented with a solution of 100 units ml^{-1} penicillin and 100 µg ml^{-1}streptomycin (Invitrogen). The muscle, vascular tissue and glands were then removed and the trachea cut into three to four rings. Each fragment was washed in phosphate-buffered saline (PBS) and then incubated in Dispase (BD Biosciences) at 7.5 Caseinolytic Units in PBS (total volume 450 µl per trachea) for 25 min at room temperature (RT). Tracheal fragments were then transferred into ice-cold DMEM:F12 and the sheets of epithelial tissue were peeled off. The epithelial sheets and medium were transferred to an ice-cold 1.5 ml tube, and pelleted twice at 500 *g* for 3 min with a PBS wash in between. The pellets were re-suspended in 0.05% TE (Invitrogen) supplemented with 5 mM EDTA for 30 min at 37 °C. 0.5 ml of DMEM:F12 supplemented with 5% foetal bovine serum (FBS) was added to stop the reaction. The cells were pelleted (500 *g*, 3 min), re-suspended in MTEC/Plus media and plated on 24-well tissue culture inserts (BD Falcon) in MTEC/Plus media at approximately 5 × 10^4 cells per insert. The inserts were previously coated by adding 70 µl of a solution of 50 µg ml^{-1} rat tail collagen I (BD Biosciences) in 0.02 M acetic acid and leaving the solution overnight for the acetic acid to evaporate.

10.1.3. Cell maintenance

All cells were cultured in a humidified incubator at 37 °C with 5% CO_2 and all media were changed every 2-3 days. All cells (except where otherwise indicated) were maintained at sub-confluence in 10 cm tissue culture plates in 8 ml medium. Immortalised cells were passaged as follows: the cells were washed twice in phosphate buffer saline (PBS) and incubated in 0.05% TE (Invitrogen) until detached. The reaction was stopped with full medium at

twice the volume of TE solution used. The resulting cell suspension was then collected and centrifuged at 1000 RPM for 3 mins. The pellet was re-suspended in fresh medium and aliquoted into new cell culture plates. MTECs were cultured at confluence and never passaged.

MDCK cells were cultured in DMEM (21885; Invitrogen) supplemented with 10% FBS (Invitrogen). The MDCK *scrib*^{KD} cells were cultured with the addition of blasticidin at 50 μg ml^{-1} (Sigma) and G418 800 μg ml^{-1} (Invitrogen). MDCK cells were passaged twice a week at approximately a 1:10 ratio.

EpH4 cells and HEK293T cells were cultured in DMEM (21885; Invitrogen) supplemented with 10% FBS, (Invitrogen). The EpH4 cells were passaged twice a week at a 1:20 ratio, and HEK293T cells were passaged three times a week at approximately a 1:8 ratio.

16HBE cells were cultured in DMEM/F12 (31330-038, Gibco) with 10% FBS (Invitrogen) and passaged once a week at a 1:6 ratio.

The MTEC cells were cultured in MTEC/Plus media consisting of: DMEM:F12 basal media supplemented with a solution of 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, 10 μg ml^{-1} insulin (Invitrogen), 5.5 μg ml^{-1}transferrin (Invitrogen), 6.7 μg ml^{-1} selenium (Invitrogen), 0.1 μg ml^{-1} cholera toxin (Sigma), 25 ng ml^{-1} epidermal growth factor (R&D Systems), 30 μg ml^{-1} bovine pituitary extract (Invitrogen), 5% FBS, 15 mM HEPES and 0.01 μM freshly added retinoic acid (Sigma). 250 μl of medium was added to each insert and 560 μl medium to each wells.

10.1.4. Inhibitors and treatments

Inhibitors and treatments were used as specified below:

Treatment	Function	Stock	Concentration used	Catalogue No./Brand
SB202190	MAPK p38 inhibitor	10 mM in DMSO	10 μM	559388/ Calbiochem
Pifithrin-α	p53 inhibitor	10 mM in DMSO	10 μM	P4359-5MG/ Sigma
Y27632	ROCK inhibitor	10 mM in H_2O	30 μM	Y0503-1MG/ Sigma
PP2	Src inhibitor	50 mM in DMSO	Depending on experiment	P0042-5MG /Sigma
Mn^{2+}	Activator of integrins	10 mM in H_2O	Depending on experiment	M3634-100G-D /Sigma
Gd^{3+}	Inhibitor of Piezzo	100 mM in H_2O	100 μM	439770-5G /Sigma
JTE013	S1P2 inhibitor	100 mM in DMSO	10 μM	2392 /Tocris Bioscience
SKI II	Sphingosine Kinase	100 mM in DMSO	30 μM	567741-5MG/ Calbiochem
Tetracycline	Induces construct expression	10 mg/ml in H_2O	5 μg/ml (10 μg/ml for RNAseq)	T7660/ Sigma
Nutlin-3	Activation of p53	10 mM in DMSO	Depending on experiment	Cayman Chemicals
Blebbistatin	non-muscle Myosin-II inhibitor	10 mM in DMSO	37.5 μM	B0560-1MG/ Sigma

10.1.5. Freezing and storing cells

All immortalised cells were frozen as follows. The cells were harvested in 0.05% TE (Invitrogen), centrifuged at 1000 RPM for 3 mins, re-suspended in 90% FBS + 10% DMSO at a million cells per 1 ml solution per cryo-vial and stored in liquid nitrogen.

10.1.6. PDMS-based cell compression assays

Cells were plated onto a stretched flexible silicone substrate (Gel pak PF-60-X4, 150 µm thickness, Teltek), held in a custom-made chamber (GREM;http://www.jove.com/video/51193/stretching-micropatterned-cells-on-a-pdms-membrane). Before plating, the clamped membranes were coated with 25 µg ml^{-1} fibronectin/PBS (Sigma) for 1 h at 37 °C. The membranes were stretched precisely by 2 cm, which provided a 57% stretch over the resting length (unless otherwise specified). A PDMS rectangular chamber, with two compartments (6.6 × 13 mm each) was placed on the membrane. Two densities (low and high) of tetracycline pre-treated or Nutlin-3 pre-treated (48 h) cells were plated, one in each compartment. High-density cells were plated to form a confluent monolayer (75,000 – 120,000 cells for MDCKs, 160,000 – 180,000 cells for 16Hbes and 200,000 – 300,000 cells for Eph4s); low-density cells were plated at sub-confluence (25,000 – 35,000 cells for MDCKs, 40,000 cells for 16Hbes and 20,000 – 35,000 cells for Eph4s). The cells were allowed to adhere for 24 h and then the membrane was released to induce compression. p38 inhibitor (SB202190; 10 µM) and ROCK inhibitor (Y27632; 30 µM) were added 1 h before releasing the membrane. Mn^{2+} treatment (10 µM) started 24 h before seeding the cells on the PDMS membrane.

The cells were fixed in 4% PFA/PBS after 1.5 h (phosphorylated-p38 staining) or after 5 h (cleaved Caspase-3 staining) from releasing the membrane, and were then processed for immunofluorescence. As per design, low- and high-density cells were stained and imaged from the same stretcher avoiding sample-to-sample variability.

Nuclear phosphorylated-p38 mean intensity was measured using Volocity (http://www.perkinelmer.co.uk/pages/020/cellularimaging/products/volocity.xhtml), using DAPI as a mask to segment the nuclei in 3D. Cell death in compression assays was quantified as the number of cleaved caspase-3

positive death events (counted manually) divided by total number of DAPI-positive nuclei (measured using Volocity).

10.1.7. Fences system

Where applicable, cell competition experiments were carried out in a 24-well plate using 'fences' (Aix Scientifics, http://www.aix-scientifics.co.uk/en/fences.html). The fences provide a silicon barrier, which divides a well into a round central compartment and a ring-shaped outer compartment. Fences are inserted directly into a well and pre-warmed to 37°C before plating cells. When the cells adhere, fences have to be removed in order to change medium or to initiate live imaging. The system allows two different populations to be spatially separated, while sharing the same media, treatments and environmental conditions. Moreover, immunofluorescent staining can be performed jointly on both populations, minimising sample-to-sample variability and thereby facilitating quantitative analysis.

10.1.8. Cell competition assays in MDCK cells

Cell competition assays on MDCK cells were carried out in 24-well plate fences. Control cultures (monocultures) were plated in the centre of the fence (1,000 cells per fence). Competition cultures were seeded on the outside of the barrier (8,000 cells per fence) at a ratio of 1:10, loser cells: winner cells. The fences were removed approximately 5 h after plating and the culture medium was replaced with fresh media. Forty eight hours later, the culture medium was replaced with phenol red-free DMEM (+10% FBS and 1% L-glutamine, Invitrogen) to improve the quality of live imaging. Chemical inhibitors were also added at this point, unless otherwise specified. Live imaging started 2–4 h after the final media change and continued for at least 50 h with regular media changes every 2 days. Where appropriate, stills from live imaging or confocal images were used to

measure cell density in competition assay. For this purpose the number of nuclei was manually counted using DAPI and/or nuclear GFP and divided by surface area, as calculated in Fiji (http://fiji.sc/Fiji).

10.1.9. Directional cell migration assays in MDCK cells

For directional cell migration assays I co-cultured unlabelled wild-type cells (followers) and GFP-labelled multinucleated leader cells. To generate multinucleated leader cells, Nls-GFP-labelled wild-type or p53-deficient MDCK cells were plated at 12,000 cells in a single well of a 12-well plate. Blebbistatin was added on the following day, left for precisely 16 hrs, and removed by washing the cultures three times in PBS. After a 4 h recovery, the cells were harvested in 0.05 TE (Invitrogen) and filtered with 20 µm filters (CellTrics) to remove large cell aggregates. I plated 50% of the recovered cells per gridded tissue culture plate (µ-Dish 35 mm Grid-500, Ibidi). Follower (unlabelled wild-type) cells were plated at 5,000 cells per gridded plate one day before seeding the leader cells, to allow to form larger clones. Live imaging started one day after all cell had been plated. The use of gridded plates allowed imaging the same cells by live-imaging and by immunofluorescence.

Presence or absence of directional cell migration was independently assessed three times (by myself, Kasia Kozyrska and Eugenia Piddini) for individual leader cells.

10.1.10. Dose-effect and homeostatic cell density assays

GFP-labelled MDCK cells were plated at 24,000 cells per well in 24-well plates. Treatments were added two days later unless otherwise specified. Live imaging started 2-4 h after the treatment, with regular media changes every 2 days. To determine optimal drug concentration (dose-effect assays) or to assess the effect of a treatment on homeostatic cell density, the

number of cells was followed over time by quantifying the number of fluorescently labelled nuclei in Fiji (http://fiji.sc/Fiji).

10.1.11. Cell competition assays in MTEC culture

The MTEC cells were plated in collagen-coated tissue culture inserts and allowed to grow for approximately 2 weeks until they reached homeostatic density before commencing experiments. In control cultures, wild-type (unlabelled) cells were plated with wild-type *Rosa*26R-Tomato (nuclear red) cells at a 2:1 ratio. In competition cultures, unlabelled p53-null cells and wild-type *Rosa*26R-Tomato cells were plated at a 2:1 ratio. Nutlin-3 was added at 17.5 μM on day 3 of live imaging. The medium was changed every 2 days.

Quantifications of cell number over time were carried out in Fiji (http://fiji.sc/Fiji), using the nuclear Tomato signal to segment cells.

10.1.12. Wounding assay in MTEC culture

Confluent MTEC cultures were wounded by scratching the monolayers with a Gilson pipette tip, and thereby creating an area free of cells. Following wounding, the cells were washed once in PBS and fresh medium was added. The cultures were fixed 8 h after the injury and processed for immunofluorescence analysis as described below.

10.2. **Molecular biology**

10.2.1. Mouse genotyping

*Rosa*26R-Tomato mice were genotyped by placing ear-clips under a light fluorescent microscope and assessing the intensity of fluorescence in the red channel. p53-null mice were genotyped using the Non-HotStart PCR Kapa Genotyping Kit (Kapa, KK7302) according to manufacturer's

instructions. The following primers targeting p53 were used: common primer (5'-TGG ATG GTG GTA TAC TCA GAG C-3'), mutant forward primer (5'-CAG CCT CTG TTC CAC ATA CAC T-3') and wild-type forward primer (5'-AGG CTT AGA GGT GCA AGC TG-3'). The following PCR program was followed:

Step #	Temp °C	Time	Note
1	94	2 min	Initial Denaturation
2	94	20 s	
3	65	15 s	-0.5 C per cycle decrease
4	68	10 s	
5	-	-	Repeat steps 2-4 for 10 cycles
6	94	15 s	
7	60	15 s	
8	72	10 s	
9	-	-	Repeat steps 6-8 for 28 cycles
10	72	2 min	Final extension
11	10	-	Hold

10.2.2. Virus production and infections

HEK293T cells were plated in 25 cm X 25 cm square tissue culture plates at 20 million cells per plate. Two days later, when 70-80% confluent, the cells were transfected as follows:

Tube A (1.5ml Eppendorf)

15 µg psPAX2 (packaging vector)

15 µg pMD2G (envelope vector)

22.4 µg pTRIPZ

Sterile water to 112 µl total

Tube B (1.5ml Eppendorf)

1.5 ml DMEM + 10% FBS

150 µl FuGENE transfection reagent (Promega)

Both tubes were mixed by flicking. The contents of tube A was then added to tube B, flicked to mix, pulse-centrifuged and incubated for 15 min at room temperature. The transfection solution was then added to the HEK293T cells in 70 ml of fresh DMEM + 10% FBS.

On the next day, the transfection medium was removed and replaced with 70 ml of fresh DMEM + 10% FBS. Following two days of incubation, the infection medium was then collected in 50 ml tubes and centrifuged for 5min at 1000 RPM to remove floating debris and cells. The medium was then transferred to 50 ml syringes and filtered through 0.45µm filters into fresh tubes.

To concentrate the virus, cold (4°C) PEG-it Virus Preparation Solution (5X) was added at 1:4 to the virus medium and refrigerated overnight. The mixture was then centrifuged at 1500 g for 30 min at 4°C to pellet the virus. After removing the supernatant, the pellets were re-suspended in fresh medium at 1/20 to 1/50 of the original volume, depending on the experiment. The concentrated virus was then aliquoted into cryovials and stored at -80°C.

MDCK cells were infected by adding 0.5 ml of the concentrated virus per well of a 24-well plate. After a 24 h incubation the virus medium was removed, the wells were washed three times in PBS and 1 ml of fresh DMEM +10% FBS was added.

10.2.3. Sequencing p53 in mutant MDCK cell lines

Wild-type MDCK cells and cells mutagenized by CRISPR were cultured in 6-well plate, harvested in 0.05% TE (Invitrogen) and centrifuged at 1000 RPM

for 3 mins. Genomic DNA was then extracted from the resulting pellet using the DirectPCR Lysis Reagent (Viagen Biotech) supplemented with Proteinase K (New England BioLabs) at 50 µl of Proteinase K per 1 ml of lysis reagent. Cells harvested from a single well of a 6-well plate were re-suspended in 140 µl of lysis solution, incubated overnight at 55°C and for 45 min at 85°C. DNA concentration was measured with NanoDrop.

A fragment of 516 bp containing the mutagenized site was then amplified with the Phusion High-Fidelity DNA Polymerase (New England BioLabs) as per manufacturer's instructions, with the following primers: FW (5'-TGCTCTCATCTTCCAGGCTT-3') and REV (5'-GAGGCCAAAGGTCACAGAAT-3'). The following PCR protocol was followed:

Step #	**Temp °C**	**Time**	**Note**
1	98	30 s	Initial Denaturation
2	98	10 s	
3	56	30 s	
4	72	30 s	Repeat steps 2-4 for 30 cycles
5	72	10 min	Final Extension
6	4	-	Hold

The resulting fragments were separated on a 1% agarose gel, recovered with the QIAquick Gel Extraction Kit (Qiagen) and sub-cloned with the Zero Blunt® TOPO® PCR Cloning Kit for Sequencing (Invitrogen) and processed as per manufacturer's instructions. 10 clones per sample were then sequenced using T3 primer.

10.3. **Imaging**

10.3.1. Immunofluorescence

For immunofluorescence, MDCK cells were cultured on glass coverslips or on gridded dishes (µ-Dish 35 mm Grid-500, Ibidi). The cells were fixed for 10 min in 4% PFA/PBS, quenched for 10 min in 50 mM NH_4Cl/PBS and then

permeabilised for 10 min with 0.1% Triton X-100/PBS. The cells were blocked in 2% BSA, 2% FBS/PBS for 30 min. Primary and secondary antibodies were diluted in blocking solution diluted 1:1 in PBS. The primary antibodies were incubated for a minimum of 1 h at RT, followed by washes in PBS; secondary antibodies were incubated for a minimum of 30 min at RT followed by washes in PBS. Coverslips were mounted with FluorSave (Millipore). For immunostaining against phosphorylated proteins, fixing solution was supplemented with PhosSTOP (1 tablet per 10 ml, Sigma), all PBS solutions were substituted with TBS, and blocking solution was substituted with 5% BSA/TBS. For surface immunostaining, the cells were washed in ice-cold phenol red-free DMEM (Gibco) and incubated with primary antibody diluted in ice-cold phenol red-free DMEM at 4 °C for 45 min. The cells were then washed in ice-cold PBS before fixation at RT in 4% PFA/PBS for 10 min. Secondary antibody staining was then carried out as outlined previously.

Throughout immunostaining, MTECs remained attached to the porous membrane on which they were cultured. MTECs were fixed in 4% PFA/PBS for 10 min at RT. The cells were penetrated with 0.1% TritonX-100/PBS for 10 min at RT and blocked in 3% BSA + 10% FBS in 0.1% TritonX-100/PBS for 1 h at RT. Primary antibodies were diluted in blocking solution and incubated overnight at 4°C. Secondary antibodies were prepared in 5% FBS in PBS and incubated for 1½ h at RT. The membranes were mounted with FluorSave (Millipore).

10.3.2. Antibodies:

I used the following primary antibodies:

Target	Specie	Concentration	Catalogue No./Brand
p53	Rabbit	1:750	9382/Cell Signaling Technology
p21	Rabbit	1:200	Sc-397/Santa Cruz
P-p38 MAPK (T180/Y182)	Rabbit	1:50	9215/Cell Signaling Technology
P-Myosin II light chain (phospho S20)	Rabbit	1:100	Ab2490/Abcam
P-MYPT1 (Thr 853)	Goat	1:50	Sc-1732/Santa Cruz
Cleaved Caspase-3	Rabbit	1:200	9661s/Cell Signaling Technology
P-Src	Rabbit	1:100	44-660G /ThermoFisher
Vinculin	Mouse	1:500	MAP3574/Millipore
Acetylated Tubulin	Mouse	1:250	24610/Abcam
KRT-5	Rabbit	1:500	PRB-160P-100 /Covance

All primary antibodies were used with Alexa Fluor conjugated secondary antibodies (1:500, Invitrogen). DAPI (1 μg ml^{-1} Invitrogen) was used to stain the nuclei. Alexa Fluor-568 and Alexa Fluor-647 conjugated Phalloidin (1:40, Invitrogen) were used to stain filamentous actin.

10.3.3. Imaging and image analysis

Fixed samples were imaged with a Leica SP5 or SP8 confocal microscope. Unless otherwise specified, all confocal images are maximum intensity projections of multiple z sections in x/y.

For live imaging, the cells (kept at 37 °C and 5% CO_2) were imaged using a Nikon BioStation CT with a × 10 air objective with imaging frequency between every 1 h and every 6 h, with media changes every 2–3 days. For each live imaging experiment, at least five fields were imaged by time lapse and analysed.

Images were processed using Fiji, Volocity and Adobe Photoshop.

10.4. **Transcriptional profiling**

10.4.1. Preparing mRNA libraries

MDCK cells were plated at 1.3 million cells/10 cm plate, with tetracycline added at 10µg/ml immediately after plating. 67 hours later, cells were harvested with 5 ml 0.05% TE (Gibco) supplemented with 5 mM EDTA. When detached, the reaction was stopped with 5 ml of full medium and the cells were centrifuged at 1000 RPM for 3 min and rinsed with PBS without disturbing the pellet. To stabilise RNA, the pellet was then dissolved in 1 ml TRIzol (Thermo Fisher Scientific) and collected. After adding 500 µl of chloroform, the mix was vigorously shaken and left for 5 min at room temperature for the phases to separate, followed by a 15 min centrifugation at 12,000 *g*, 4°C. The solution separated into 3 phases. 0.5 ml of the top, colourless phase containing the RNA was collected and an equal volume of 70% ethanol was added. To isolate the RNA, 0.7 ml of the resulting mix was then processed with the RNeasy Mini Kit (Quiagen) supplemented with the RNase-Free DNase Set (Quiagen), as per manufacturer's instructions. The RNA was stored at -80°C.

RNA content was quantified with NanoDrop and RNA HS Assay kit (Qubit). RNA quality was assessed using the Bioanalyzer RNA 6000 Nano Kit (Agilent).

RNAseq libraries were prepared from 1 µg RNA with the TrueSeq RNA sample preparation V2 kit (Illumina) according to the manufacturer's

instructions. DNA content was measured with the DNA BR assay (Qubit). The quality of the libraries was assessed using the Bioanalyzer High Sensitivity DNA kit (Agilent). Additionally, to avoid contamination with larger DNA fragments, DNA of 180-465 bp were selected with Pippin Prep (Sage Science).

10.4.2. RNA-seq and differential expression analysis

RNAseq libraries were sequenced on an Illumina HiSeq 2000 instrument in single-read mode at 36 or 40 base length. The resulting fastq files were filtered for low-quality reads (<Q20) and low-quality bases were trimmed from the ends of the reads (<Q20).

The following procedures were carried by Charles Bradshaw and George Allen from the Gurdon Institute. Genome-based RNA-seq mapping was carried out using *Canis lupus familiaris* 3.1 (NCBI/Dog Sequencing Consortium) as a reference genome. Transcript sequences were assigned to genome using BLAT (Kent 2002). The resulting mappings were filtered by a mismatch threshold (2%), as well as requiring 90% of the transcript to match the genome and all exons to match a single chromosome. This resulted in 21,571 transcripts mapping to the genome. This mapping was used as a junction file for Tophat 2 (Trapnell *et al*. 2009), which was used to map the RNA-seq reads to the genome. To provide gene names, transcript sequences were downloaded from the NCBI RefSeq database in March 2013 (24,538 sequences). Orthologues were found against the *Mus musculus* proteome (downloaded in January 2013—NCBI RefSeq) using Inparanoid (Alexeyenko *et al*. 2006). For differential expression, read counts were generated by quantifying overlaps with transcript locations. These were then used to generate RPKMs. Comparisons were made between pairs of conditions, each with at least four replicates. For a transcript to be included, counts per million had to be above 10 for all samples in at least one condition and within 2-fold between replicates. Differentially expressed transcripts were then called using EdgeR (Robinson

et al. 2009). Hits were selected applying the following thresholds: $P<0.05$, log FC (fold change) >0.5.

I then identified Gene Ontology terms over-represented among these lists, using David Bioinformatics Resources (Huang *et al*. 2009), in particular KEGG pathway analysis (Kanehisa & Goto 2000; Kanehisa *et al*. 2012).

10.5. **Statistical analysis**

No statistical methods were used to predetermine sample size. Every experimental condition and treatment was carried out alongside a complete control set of experiments or no treatment control. The sample size was chosen to see a statistical difference between data sets. In the few instances where no difference was observed, sample size was at least as big as in conditions that had shown a difference. The experiments were not randomized and there was no blinding during experiments or analysis, as samples were marked. I carried out a minimum of independent three repeats for each experiment, unless otherwise specified.

The non-parametric KS test was used for all statistical tests, removing the requirement for normally distributed data and equal variance. Throughout: *P<0.05, **P<0.005, ***P<0.0005.

ABBREVIATIONS

4-OHT	4-hydroxytamoxifen
Act-tub	Acetylated Tubulin-α
APC	Adenomatous polyposis coli
BLAT	BLAST-like alignment tool
BMPs	Bone morphogenetic proteins
bp	base pair
Bst	Belly spot and tail
Cdk4	Cyclin-dependent kinase 4
CRISPR	Clustered regularly interspaced short palindromic repeats
CycD	Cyclin D
CySC	Somatic cyst stem cell
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA-binding domain
dlg	Discs large
DMSO	Dimethyl sulfoxide
Dpp	Decapentaplegic
FA	Focal adhesion
FC	Fold change
FBS	Foetal bovine serum
FGF	Fibroblst growth factor
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KRT	Keratin
KS	Kolmogorov–Smirnov test
lgl	Lethal giant larvae
MAPK	Mitogen-activated protein kinases
Mdm2	Mouse double minute 2 homolog

MTEC	Mouse tracheal epithelial cell
MYPT1	Myosin phosphatase target subunit 1
Nls	Nuclear localization sequence
P-	Phosphorylated
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PDMS	Polydimethylsiloxane
PFA	Polymerase chain reaction
PI3K	Phosphoinositide 3-kinase
RNAi	RNA interference
RNAseq	RNA sequencing
ROCK	Rho-associated protein kinase
RPKM	Reads Per Kilobase of transcript per Million mapped reads
RPM	Revolutions per minute
RT	Room temperature
s.e.m	standard error of mean
S1P	Sphingosine-1-phosphate
S1P2	Sphingosine 1-phosphate receptor 2
scrib	scribble
SD	Standard deviation
STAT	Signal transducer and activator of transcription
TE	Trypsin-EDTA
TET	Tetracycline
TGFβ	Transforming growth factor beta
TNF	Tumor necrosis factor
TRR	Toll-like receptor
UV	Ultraviolet
WT	wild-type

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Supplementary Data-1.

Genes differentially expressed between wild-type cells (*scrib* ^{KD} -TET) and scribble-deficient cells (*scrib* ^{KD} +TET). FC = fold change.

Transcript identifier	Official gene name	FC (*scrib* ^{KD}-TET/*scrib* ^{KD}+TET)	P value
XM_847751.2	Ripply1	13.09	9.0124E-156
XM_533124.2		12.14	3.1795E-109
XM_541459.3	Siglecg	9.82	1.54485E-64
XM_003434416.1	Vcan	8.72	3.5532E-197
AB738915.1	Slc22a12	7.00	3.0554E-111
NM_001003282.1		6.43	1.3142E-119
XM_535946.3	Lrp2	6.32	1.3137E-106
XM_546890.3	Cdh16	6.15	1.57229E-69
X14479.1		6.11	5.67814E-62
XM_546585.3	Ybx2	5.89	3.29007E-50
XM_536715.3	Samd11	5.42	5.61226E-48
XM_542992.2	D630003M21Rik	5.41	1.17086E-84
XM_532430.3	Tspan33	5.08	3.7268E-125
XM_003434612.1	Podn	4.88	7.24034E-93
NM_001197143.1	Pck1	4.85	1.95359E-79
XM_536475.4	Fst	4.83	4.63303E-69
XM_541314.3	Gadd45g	4.69	1.68468E-57
XM_539961.3	Ido2	4.59	6.027E-117
DQ138952.1		4.26	8.5016E-120
XM_547730.3	Slc7a8	4.26	7.96545E-48
XM_539370.3	Plx 4	4.07	2.57206E-70
XM_548187.3	B4galnt2	4.07	8.3251E-35
AF358907.1	Cldn2	3.96	1.2546E-100
XM_538813.4	Pappa	3.82	3.66098E-47
XM_003433272.1		3.78	5.10807E-45
FR775795.1		3.77	7.0475E-103
XM_546842.3	Chst4	3.74	1.7342E-101
XM_535920.3	Nr4a2	3.72	3.26974E-29
XM_844250.2		3.71	1.33506E-54
XM_548371.3	Egfl7	3.70	5.4086E-109
XM_542303.3	Dgat2	3.63	1.54381E-45
NM_001003227.1	Nr4a1	3.58	1.02811E-39
XM_533818.3	Zmynd10	3.53	1.2377E-30
AB191461.1		3.48	1.05279E-69
AB240641.1		3.48	1.16077E-66
AF045773.1	Adm	3.46	5.5797E-65
U96127.1	Adm	3.46	4.52766E-62
AB240642.1		3.42	4.56768E-65
XM_535525.3		3.42	5.60902E-28
XM_537133.3	Pigr	3.40	6.88262E-31
XM_533253.3	Pla2g16	3.33	2.88831E-39
XM_533874.3	Ifi30	3.18	2.65857E-81
XM_844782.2	Rdh10	3.15	4.13608E-98
XM_003433436.1	Gstt2	3.13	1.40418E-89

AY081057.1		3.13	1.64299E-21
NM_001252337.1	Fxyd2	3.12	8.431E-67
XM_844686.1	Fam167a	3.11	4.54258E-27
XM_003432847.2	Palm3	3.07	3.55672E-39
XM_003433751.1	Sh2d5	3.06	2.21939E-35
XM_531781.2	Npas2	3.02	2.72373E-28
AB031064.1	S100a4	2.98	1.60007E-56
XM_540097.1	Osr1	2.97	6.05026E-47
NM_001195154.1		2.96	3.96535E-70
XM_003433456.1	Fam211b	2.95	2.70109E-30
XM_003434496.1		2.93	3.07631E-48
XM_536010.3	Tmeff2	2.92	2.38927E-72
AF023617.1	Tjp3	2.89	4.03678E-67
XM_844867.2	G l	2.88	8.80519E-25
XM_003639180.1	Cenpf	2.87	1.08108E-83
XM_849019.2	Msln	2.87	8.59342E-43
XM_542817.3	Pls1	2.86	3.72684E-53
XM_003639229.1	Cdh24	2.84	4.2659E-49
XM_544886.3	Ets2	2.81	2.33464E-40
XM_546705.3	Cpt2	2.81	7.91976E-82
XM_536357.3	Phyhipl	2.75	8.70839E-78
XM_848972.2	Chrm4	2.74	2.34594E-69
XM_547265.3	Frrs1	2.74	2.12259E-78
XM_546603.1	Alox8	2.73	5.09909E-46
XM_850298.2	Klhl14	2.72	9.98157E-25
AF178116.1		2.69	4.25893E-64
XM_003639522.1	Tspan1	2.68	4.94755E-22
XM_846805.2	Fam110b	2.66	1.00283E-62
XM_003433355.2	Hjurp	2.66	1.8857E-72
XM_533037.3	Txnip	2.64	7.78381E-59
XM_847332.2	Pcbd2	2.63	2.91986E-28
XM_543238.3	Stc1	2.59	2.61684E-69
XM_546209.3	Mxd3	2.58	2.64982E-33
D29807.1	Sult1a1	2.56	6.19788E-39
XM_538109.3	Gla	2.55	2.08421E-28
XM_545936.3	Fgfbp1	2.55	1.01009E-17
XM_003432240.1		2.55	1.91325E-35
XM_003639179.1	Mfsd7b	2.55	6.60082E-48
XM_535535.3	Hcn4	2.55	8.77527E-19
XM_003433723.1	Nt5c2	2.53	3.61117E-59
XM_846096.2	Desi2	2.53	2.31131E-21
XM_849464.1		2.52	1.23113E-27
XM_851385.2	Vim	2.50	2.13598E-57
XM_003640151.1	Btn1a1	2.50	3.55008E-22
XM_003433118.1	Plch1	2.49	6.42674E-25
NM_001146269.1	Cdkn2b	2.49	1.11727E-24
XM_547272.3	Tmem56	2.49	3.04507E-32
XM_546637.2	Trim16	2.48	5.16112E-31
AY064408.1		2.48	1.35335E-27
EF432321.1		2.47	6.54159E-64

XM_543194.3	Hmgb2	2.47	6.86734E-61
XM_847879.2	Tmem37	2.47	1.21469E-37
XM_535839.2	St6gal1	2.47	2.67939E-35
XM_533525.3	Aldh1a1	2.47	2.79357E-65
XM_003432414.1	Cntf	2.46	1.58827E-18
AY069922.1	Sult1a1	2.44	4.24151E-59
XM_536655.3	Zswim7	2.43	6.81895E-27
XM_532560.4	Oscp1	2.43	5.52766E-26
XM_539692.4	E2f7	2.42	1.90386E-26
XM_540076.3	Rrm2	2.42	4.35795E-66
XM_535331.3	Fabp3	2.42	2.9904E-37
XM_548435.3	Wdr34	2.42	3.77673E-41
XM_548195.3	Pdk2	2.40	1.53259E-51
XM_541746.3	Nup210	2.40	4.22333E-16
XM_849870.1	Wnt2	2.39	2.55203E-20
XM_548096.3	Ttc25	2.37	8.84895E-22
XM_847234.1		2.37	1.75233E-30
XM_853642.2	Ap3s1	2.37	1.00796E-33
XM_546160.4	Mcu	2.36	7.22335E-48
XM_003431819.1	Scrib	2.36	7.37436E-49
XM_843265.3	Gdf11	2.36	2.33943E-22
NM_001031816.1	Sstr1	2.36	6.24516E-34
XM_849866.2	Anks6	2.36	2.10088E-31
XM_538268.3	Tbc1d30	2.35	1.00636E-27
NM_001197189.1	Slc35c1	2.35	1.15413E-39
XM_844405.2	Rhobtb1	2.35	3.65037E-46
XM_541943.3	Mpv17l2	2.35	8.95499E-48
AY305400.1		2.34	3.83695E-28
XM_532589.3	Pdzk1ip1	2.34	1.52624E-40
XM_532891.3	Ncoa1	2.34	6.2939E-27
XM_003432490.1		2.34	7.75737E-18
XM_548423.3	Ier5l	2.34	1.44702E-42
XM_543918.2	Slc16a12	2.34	1.20671E-45
DQ784645.1	Slc11a1	2.33	1.21164E-22
XM_848544.1	Tmed6	2.33	3.44335E-21
XM_003432071.1	3110062M04Rik	2.33	2.41342E-30
NM_001145170.1	Car2	2.32	6.50109E-53
XM_847037.2		2.31	7.99613E-27
XM_858603.3	Foxp1	2.30	1.16004E-27
XM_003431808.1	Ly6e	2.29	1.37244E-46
XM_003432527.1	Myb	2.28	2.25195E-17
XM_535923.3	Ccdc148	2.27	8.50749E-42
XM_003435109.1	Nfatc4	2.27	8.94008E-28
XM_850100.3	Arid3a	2.27	3.51964E-25
XM_003432588.1		2.27	1.52553E-39
XM_543039.3	Ncoa3	2.26	7.13648E-30
XM_845175.2	Cldn16	2.26	1.96011E-22
XM_859074.2	Bmp4	2.26	5.66782E-41
XM_003433010.1	Stard10	2.25	1.62328E-20
XM_533210.3	Chka	2.25	3.29161E-29

XM_854718.2	3110062M04Rik	2.24	7.16532E-29
XM_848770.2	1700011H14Rik	2.24	2.97071E-49
XM_542979.3	Epb4.1l1	2.23	2.97021E-28
XM_540139.3	Capn13	2.23	1.91146E-27
XM_547195.3	Ift140	2.23	7.99146E-26
AY587107.1	Slc29a1	2.23	1.9629E-28
EF063141.1	Ptges	2.23	1.01907E-24
XM_533692.3	Tmem147	2.22	6.22486E-41
NM_001013844.1	Fosb	2.22	5.29492E-22
XM_543443.3	Dao	2.22	2.5095E-42
XM_538677.3	Mllt3	2.22	8.52308E-31
XM_853571.3	Tenm4	2.21	1.91074E-09
DQ138951.1	Cyp4a10	2.20	7.04582E-38
XM_542865.3	Foxa2	2.20	1.5561E-30
XM_003432778.1	Slc44a2	2.19	1.94149E-43
XM_543220.3	Zfp395	2.19	2.81307E-18
XM_848163.2	H2afx	2.19	6.74129E-21
XM_003433995.2	Adamts1	2.18	4.76687E-19
XM_547533.2	Iqgap3	2.18	6.69566E-38
XM_003431466.1	Cpm	2.18	2.69445E-27
XM_537238.3	Cnih4	2.16	4.61923E-32
XM_849853.2	Sobp	2.15	3.22525E-18
XM_536595.3	Pdgfd	2.14	3.72934E-17
XM_850256.3		2.14	1.34666E-33
XM_549177.3	Tsc22d3	2.13	2.91988E-32
XM_534912.2	Scnn1a	2.13	1.60872E-17
XM_532487.1	Itgb8	2.13	6.61862E-44
XM_547577.3	Npr1	2.13	3.51567E-12
XM_534792.4	Tenc1	2.13	8.39879E-21
XM_543403.3	Rasal1	2.12	4.35142E-37
XM_003433593.2	Mki67	2.12	2.732E-30
XM_544383.3	Mmp15	2.11	1.37701E-23
XM_003640219.1	Med12	2.10	4.53014E-30
XM_540754.2	Ambra1	2.10	1.90241E-35
HQ412642.1	Havcr1	2.10	7.69538E-36
XM_846951.2	Kctd2	2.10	1.1082E-24
XM_543682.3	Kcnh3	2.09	1.39739E-17
XM_847350.3	Hoxd9	2.09	8.64639E-14
XM_003432423.1		2.09	1.3152E-14
AY156692.1	Ctss	2.09	1.18762E-28
FR775794.1		2.09	9.49819E-21
XM_856952.2	Hnrnpa3	2.09	6.28289E-44
AF345933.1		2.08	2.86335E-24
AF211257.1	Fgfr2	2.08	6.57227E-41
XM_863075.2		2.08	1.41458E-34
XM_003435269.2	Tob1	2.07	8.10253E-24
XM_003638813.1	Atf5	2.07	1.03512E-25
XM_547914.3	Fos	2.07	6.99517E-34
XM_544123.2	Trpa1	2.07	9.72284E-41
XM_539383.3	Irf5	2.07	1.09133E-16

XM_547148.3	Glis2	2.06	1.85014E-15
XM_543166.3	Spata13	2.06	1.58002E-18
XM_845609.2	Cbx4	2.05	8.04352E-36
XM_531981.3	Dctn3	2.05	2.81788E-33
XM_545778.3	Olfml2b	2.04	6.33285E-38
XM_548055.3	Kif18b	2.03	1.90434E-19
XM_539384.3	Kcp	2.03	1.50005E-19
XM_535560.2	Nrip1	2.03	4.76801E-23
XM_846419.2	Zfp362	2.02	4.37926E-27
XM_545023.3	Elovl6	2.02	2.0298E-42
AF209748.1		2.02	3.47082E-13
AB049597.1	Egf	2.02	1.88129E-27
XM_003639845.1	Ptgis	2.02	6.28272E-21
NM_001172543.1	Peg10	2.02	3.8013E-27
EF427641.1	Arhgap1	2.01	1.32165E-28
XM_546495.3	Bcl9l	2.01	6.01262E-18
AY422569.1	Unk	2.00	2.14038E-28
XM_003432893.1	Ssbp4	2.00	1.37033E-29
XM_003639062.1	Spire2	2.00	8.48625E-23
XM_537333.2	Apcdd1	2.00	1.11768E-25
XM_849357.1	Bcas1	1.99	1.17873E-16
XM_846724.1	Tspan15	1.99	3.06529E-19
XM_533618.4	Fcgrt	1.99	8.32698E-37
XM_542216.4	Palm	1.99	1.11693E-19
XM_533084.3	Vps41	1.98	1.1689E-36
XM_848422.2	Dcdc2a	1.98	9.8886E-37
XM_548234.3	Rnf43	1.97	4.52778E-21
XM_852973.2	Prkar2b	1.97	3.04356E-19
XM_540546.3	Ehf	1.97	3.13433E-32
XM_544403.3	Irx3	1.97	6.02539E-27
DQ138950.1		1.97	2.45063E-38
XM_541159.3	Cnksr3	1.97	4.33696E-17
XM_844501.2	Slx1b	1.97	6.58528E-34
XM_540827.3	Sptbn2	1.96	6.07035E-28
XM_850106.2	Rap1gap	1.96	1.66577E-23
XM_003432104.1	Kat6a	1.96	8.41624E-25
XM_849496.2	Tmem54	1.96	3.97167E-17
XM_003433441.1	Med15	1.96	3.68605E-27
XM_539903.3	Zfp467	1.96	4.64988E-13
XM_852283.2	Acsl1	1.95	5.56489E-39
XM_003432501.1		1.95	2.65143E-25
XM_003431591.1	Isoc1	1.95	4.64643E-20
XM_545055.3	Stx19	1.95	2.34968E-18
XM_003433626.1		1.94	3.70821E-33
NM_001252172.1	Nde1	1.94	4.89534E-17
XM_544159.2	Car13	1.93	1.55018E-28
XM_003431870.1		1.93	3.02091E-11
XM_849871.2	Macrod1	1.93	9.18736E-22
XM_003434597.1	Fam211a	1.92	2.63863E-19
XM_532008.4	Galnt12	1.91	1.47534E-20

XM_847302.2	Mxi1	1.91	1.44121E-27
XM_546757.3	Slc45a1	1.91	1.76064E-17
XM_539487.3	Hoxa5	1.91	8.81357E-27
XM_549171.3	Rnf128	1.91	5.50899E-10
XM_539381.2	Smo	1.91	4.53311E-28
XM_544912.4	Sik1	1.90	5.99081E-16
AJ833648.1	Ccnb3	1.90	3.50986E-12
XM_003434733.2		1.90	1.32006E-18
XM_843234.2	Ankrd10	1.90	2.97515E-20
XM_844215.2	Sytl2	1.90	1.14447E-26
XM_845717.2	Sdf2l1	1.90	1.72416E-31
XM_003639145.1		1.90	8.13126E-18
XM_545160.3	Bdh1	1.90	1.27925E-19
XM_542889.2		1.89	2.06106E-24
XM_003435638.1	Rnf128	1.89	1.03593E-13
XM_534119.3	Itm2b	1.89	5.90029E-33
XM_849544.2	Marcksl1	1.88	4.06296E-24
XM_848380.1	Il10ra	1.88	3.22891E-12
XM_846751.2		1.88	5.3023E-16
XM_537051.3	Extl2	1.88	1.07288E-14
XM_542172.4	Pip5k1c	1.88	2.19327E-15
XM_546493.3		1.88	1.74607E-15
XM_849432.2	Tbp	1.88	6.25621E-19
XM_003639105.1	Gprc5b	1.87	3.47375E-31
XM_848450.2	Sgpp2	1.87	2.32743E-14
XM_537031.3	Dram2	1.87	2.11345E-23
XM_846988.2	Pcbd1	1.87	1.76317E-23
XM_547695.3	Zbtb7c	1.87	1.65053E-14
XM_858078.2	Gm15453	1.87	1.34785E-28
XM_539217.3	Slc39a4	1.87	3.26272E-22
XM_848277.3		1.87	2.33946E-22
XM_546176.3	Zfp503	1.87	9.80524E-14
XM_845170.2	Zc3h12a	1.86	1.60597E-13
XM_534985.4	Hps1	1.86	8.08596E-24
XM_848041.3	Mcm5	1.85	7.8809E-30
XM_533747.3	Arpc4	1.85	1.14604E-32
XM_859451.1	Hoxa3	1.85	5.81733E-17
XM_537413.3	Nfkbia	1.84	8.05552E-22
XM_849647.2	Ppp1r14c	1.84	2.98045E-18
XM_850427.3		1.84	1.96433E-11
XM_538901.3	Kcnk5	1.84	2.88006E-18
XM_547874.3	4933426M11Rik	1.84	8.00141E-19
XM_848710.2	1110008J03Rik	1.84	4.02335E-21
XM_848818.1	Spc24	1.84	7.90702E-22
XM_547007.3	Iqce	1.83	1.39679E-16
XM_541624.3	Zfp36	1.83	1.0626E-19
XM_543240.3	Nkx3-1	1.83	3.89968E-14
AF167075.2	Slc1a1	1.83	1.59939E-30
XM_545193.3	Slc12a7	1.83	1.07345E-29
XM_003433829.1	Pllp	1.83	5.25978E-30

XM_533451.3	Akap12	1.83	3.14662E-32
XM_543950.2		1.83	1.86221E-15
XM_534969.3	Rbp4	1.83	2.30569E-30
XM_534845.3	2810474O19Rik	1.83	2.66803E-30
XM_003640179.1		1.83	1.20683E-08
XM_532021.3	Nips p3b	1.83	1.65618E-20
XM_540446.3	Rnf157	1.83	3.31747E-16
XM_533626.3	Ppp1r15a	1.82	9.80306E-28
XM_003639190.1		1.82	8.39097E-13
XM_845674.2	Hyal1	1.82	3.01959E-27
XM_542966.3	Chmp4b	1.82	1.7495E-29
XM_844466.2		1.82	7.24517E-25
XM_003435293.1		1.82	1.0615E-22
XM_846105.1	Irx5	1.81	4.23379E-19
XM_536817.3	Zdhhc1	1.81	4.22097E-19
XM_003433177.1		1.81	3.26171E-16
XM_548392.3	Rexo4	1.81	4.21642E-28
XM_532713.3	Klhl2	1.81	4.2471E-21
XM_003435525.1	Ccdc160	1.81	6.88981E-15
XM_541860.3	Abhd14b	1.81	6.29127E-23
XM_847901.2	Hnf1b	1.81	3.44263E-12
NM_001253903.1	Poldip2	1.81	7.40755E-28
XM_534128.3	Tsc22d1	1.81	1.99362E-13
XM_003432667.1		1.81	5.03107E-28
XM_534901.3	C1ra	1.80	1.08817E-18
XM_547221.4	Itfg3	1.80	3.58052E-20
XM_003432073.1	Ezh2	1.80	3.53938E-20
XM_844184.2	Upk3b	1.80	2.46504E-12
XM_847046.1	Itprip	1.79	5.30326E-22
XM_544474.3	Fam46b	1.79	3.6125E-10
XM_003432648.2	Vsig10l	1.79	1.00371E-08
XM_847921.1	Fa2h	1.79	2.818E-12
XM_540908.3	Eml3	1.79	2.31263E-24
XM_534757.3	Prodh	1.79	2.6744E-16
XM_542613.4	LOC101056336	1.79	2.07627E-19
XM_536247.3	Nsun7	1.79	1.89216E-16
XM_855278.2		1.79	3.83655E-10
XM_535163.3	Spag6	1.79	1.42562E-19
XM_003639406.1		1.78	5.93283E-18
XM_843188.1	Cebpd	1.78	1.32683E-12
XM_844504.2	Tfdp1	1.78	2.99967E-19
XM_543406.3	Slc24a6	1.78	2.76533E-11
XM_536993.3	Cluap1	1.78	4.78388E-12
XM_546150.3	Sgpl1	1.78	3.55372E-20
XM_533114.3	Cdc42ep4	1.78	3.68508E-19
XM_540198.3	Retsat	1.78	9.22289E-28
XM_545146.3	Muc20	1.77	8.22103E-19
XM_845177.2	Igfbp7	1.77	1.11869E-27
XM_532856.4	Tssc1	1.77	1.45294E-26
XM_849320.2		1.77	9.19955E-25

XM_546525.3	2310030G06Rik	1.77	8.93223E-17
XM_540457.3	Sec14l1	1.76	3.66389E-22
XM_534994.3	Dpcd	1.76	3.34182E-16
XM_542208.3	Abca16	1.76	4.87971E-10
XM_003432169.1		1.76	2.50631E-12
XM_003639801.1	Tsc22d1	1.76	4.21787E-24
XM_546111.3	Ccdc6	1.76	1.79971E-15
XM_848253.2		1.76	1.56006E-15
XM_548204.3	Abcc3	1.75	1.37935E-18
XM_542330.3	Lrrc51	1.75	1.09226E-09
XM_538304.2	Mapk8ip2	1.75	5.28904E-13
XM_537795.3	Notch1	1.75	3.36311E-15
XM_538237.3	Baz2a	1.75	4.64717E-18
XM_003434415.1	Ankrd32	1.74	6.71614E-17
XM_541509.3	Bcat2	1.74	1.60779E-15
XM_847778.2	Stil	1.74	8.58952E-15
XM_003435274.1		1.74	1.62291E-26
EF094479.1		1.74	1.04256E-10
XM_538611.4	Slc12a2	1.74	1.90938E-25
XM_535843.3	Leprel1	1.74	1.35024E-26
NM_001131050.1	Ptges2	1.73	5.13915E-17
AM048627.1	Ctsd	1.73	1.85404E-25
XM_544014.3	Dusp5	1.73	8.51961E-16
XM_003638754.1	Tshz1	1.73	3.54769E-13
XM_533632.3	Sae1	1.73	1.89986E-25
XM_846654.2	Gm7367	1.73	3.2236E-24
XM_859340.2		1.72	6.01501E-11
XM_003432283.1		1.72	8.28866E-11
XM_536762.3	Atp2c2	1.72	7.70799E-16
JN656398.1		1.72	7.11857E-08
XM_544899.2	C2cd2	1.72	1.77135E-20
XM_850086.2	Tmem132a	1.72	4.07229E-10
XM_862787.2	Myrf	1.72	7.77026E-09
XM_003639660.1		1.72	5.1798E-20
XM_548092.3	Stat5b	1.72	3.59282E-21
XM_536095.3	Plekha6	1.72	7.61105E-13
XM_533391.3	Zfp532	1.72	9.08569E-20
XM_003639926.1	Lipa	1.72	8.2701E-13
XM_843432.2	Pkp4	1.72	3.335E-26
XM_849288.2	Cd81	1.72	2.81664E-23
XM_543225.3	Scara3	1.71	6.01784E-21
XM_848699.2		1.71	3.15211E-14
XM_548064.4	Hdac5	1.71	7.46797E-16
XM_846507.2	Dock11	1.71	8.19617E-18
NM_001013416.1	Brca1	1.71	1.99912E-17
XM_003435518.1	Clcn5	1.71	1.72117E-09
XM_848220.2	Hdhd1a	1.71	1.40319E-18
XM_003433866.1	Arhgef10l	1.71	7.67901E-12
XM_845545.3	Uck1	1.71	1.317E-14
XM_003435315.1	Nsmf	1.70	4.27142E-21

XM_534319.3	Mlf1	1.70	5.04193E-10
XM_845097.2	Cdca8	1.70	2.49872E-13
XM_849094.3	1810043G02Rik	1.70	1.07579E-13
XM_532917.3	Rbks	1.70	5.55694E-16
XM_849230.2	Syt8	1.70	3.87797E-18
XM_003433758.2	Diap1	1.70	5.65615E-22
XM_003432560.1		1.70	2.48856E-21
XM_538914.4	Foxp4	1.70	3.35327E-11
XM_003434864.1	Crebbp	1.70	4.86509E-14
XM_843553.3	Fam171a2	1.70	2.52938E-09
XM_533030.2	Acp6	1.70	2.90217E-25
X83591.1	Pax8	1.70	9.05807E-12
XM_861511.2	Akap1	1.70	1.27685E-23
XM_849177.2	Hnrnpm	1.69	1.4834E-24
XM_539201.3	Mapk15	1.69	9.4539E-08
XM_538224.3	Suox	1.69	1.17445E-21
XM_848366.2	Tmem125	1.69	3.39536E-16
XM_003432577.1	Erf	1.69	3.26619E-14
XM_845621.3	Mllt6	1.69	8.7542E-19
XM_543973.3	Sema4g	1.69	1.87763E-12
AY136626.1		1.69	2.61871E-14
XM_003435619.1		1.69	3.43361E-10
XM_546606.4	Per1	1.69	2.81604E-15
XM_533928.3	Angptl4	1.69	6.67899E-11
XM_848051.2	Gabarapl1	1.69	2.84016E-22
XM_849180.2	Zfp358	1.68	1.2447E-19
XM_846803.2	Mcat	1.68	1.06569E-19
XM_003640036.1	Knstrn	1.68	3.91738E-14
JN656393.1		1.68	1.52884E-13
XM_536435.3	Wwc1	1.68	2.03908E-19
XM_003639434.1	Paqr8	1.68	2.75856E-18
XM_003638814.1		1.68	4.32498E-21
XM_531697.3	Atxn10	1.68	7.83702E-23
XM_847209.2	Srf	1.68	4.69994E-16
XM_538382.3	Ankrd54	1.68	7.58438E-13
NM_001194984.1	LOC100045999	1.67	1.87713E-22
JN656391.1	Tap1	1.67	2.97242E-18
XM_543526.3	Ggt1	1.67	2.49867E-17
XM_845091.2	Igfbp4	1.67	1.5927E-20
XM_541428.4	Cnot3	1.67	6.46478E-08
XM_003434827.2	Rab11fip3	1.67	3.11985E-14
NM_001252198.1	Ptma	1.67	9.2622E-20
XM_536921.3		1.67	2.00114E-17
XM_003639007.1		1.67	1.85124E-10
XM_848485.2	Tada2a	1.67	2.15734E-22
XM_003432130.1	Mtus1	1.67	3.00069E-23
EF561643.1	Trpv4	1.67	1.57545E-17
XM_538053.2	Tsr2	1.66	1.40157E-07
XM_003639738.1	Slc25a23	1.66	2.69196E-08
XM_548141.3	Grb7	1.66	2.08013E-09

XM_547735.3	Zfhx2	1.66	3.65629E-07
NM_001003258.2	Lman2	1.66	2.22995E-22
XM_003432802.1	4930404N11Rik	1.66	1.64521E-13
XM_845361.2	Crym	1.66	6.66576E-14
XM_543481.3	Tcn2	1.66	1.31787E-22
XM_539627.3	Faah	1.66	1.14684E-16
XM_540856.3	Fam89b	1.66	2.60432E-12
XM_846871.2	4430402I18Rik	1.66	1.32762E-08
XM_003639008.1		1.66	2.89944E-10
XM_003432953.1	Crebzf	1.66	3.49415E-13
XM_003432323.1	Fam89b	1.66	2.5054E-13
XM_544737.4	Smad6	1.66	1.72842E-14
XM_847192.2	Jund	1.66	1.44732E-17
XM_845803.3	Srm	1.66	1.34781E-17
XM_003639165.1	BC034090	1.66	1.85147E-09
XM_849514.2		1.65	4.623E-14
AF056084.1		1.65	9.09437E-17
XM_003639373.1	Phf19	1.65	4.56798E-10
XM_849974.2		1.65	1.46503E-20
XM_003434924.1	Kctd1	1.65	5.42135E-08
AJ271644.1	Id3	1.65	1.20696E-16
XM_537548.3	Clmn	1.65	9.00431E-08
XM_003638757.1	Mtfr2	1.65	8.57376E-17
XM_858018.2	Mpc1	1.65	9.35485E-15
XM_536052.3	Kansl1l	1.64	9.51574E-22
XM_003433284.1		1.64	1.64968E-19
XM_846128.2	Hoxb7	1.64	1.97505E-13
XM_003639315.1	Dab2ip	1.64	3.08906E-11
XM_003432616.1	Kank1	1.64	1.85245E-16
XM_003432725.1		1.64	2.69128E-11
XM_541876.3	Hyal2	1.64	5.79629E-17
XM_003640189.1	Fzd5	1.64	2.07553E-16
XM_846897.1	Tbc1d10c	1.64	2.6186E-07
XM_534734.3	Sec14l2	1.64	8.59243E-13
XM_844894.2	Pcbp4	1.64	5.81112E-20
XM_545912.3	Dok7	1.64	9.72567E-14
XM_847454.2	Wwtr1	1.64	4.29635E-22
XM_003432467.1		1.64	2.89377E-13
XM_538343.3	A4galt	1.63	5.55777E-07
XM_542033.3	Ier2	1.63	4.03276E-18
XM_541942.3	Rab3a	1.63	7.93004E-12
XM_845376.1	Pgap1	1.63	3.41795E-15
XM_534602.3	Armc9	1.63	7.11912E-09
XM_546807.3	Osgin1	1.63	4.16965E-08
XM_849132.2		1.63	8.09215E-20
XM_848135.2		1.63	1.55844E-09
XM_543842.3	Lpcat3	1.63	1.02295E-19
XM_539058.2	Sim1	1.63	1.58464E-09
XM_538808.2	Kif12	1.63	4.48792E-17
XM_534424.3	Mybl2	1.63	2.15496E-20

XM_547541.3	Ubqln4	1.63	2.83887E-13
XM_535693.3	Pla2g12a	1.63	4.23195E-12
XM_849406.2	Sh3gl1	1.63	4.27596E-17
XM_003639993.1	Arl3	1.63	7.45709E-16
XM_003434888.1		1.63	5.55934E-17
XM_537530.3	Sel1l	1.62	2.78273E-20
AB194049.1		1.62	1.04602E-07
XM_533554.3	Spin1	1.62	3.49755E-15
XM_003432701.1	t14	1.62	4.32275E-10
XM_003433622.1		1.62	1.99562E-14
XM_546999.3	Fbxl18	1.62	4.75273E-12
XM_003435333.1	Arrdc1	1.62	5.35271E-20
XM_003434817.1		1.62	8.96196E-19
XM_003639722.1	Junb	1.62	8.2089E-16
XM_532516.3	Sept7	1.62	1.58728E-19
XM_540913.3	Fads2	1.62	5.82582E-21
XM_546979.3	Pdap1	1.62	1.80942E-18
XM_543529.3	Cabin1	1.62	1.94135E-15
XM_547146.2	Nmral1	1.62	3.28598E-19
XM_546998.3	Fscn1	1.62	6.01817E-08
XM_843429.2	Slc12a6	1.62	9.01959E-20
XM_538474.3	Plekhh2	1.61	4.14626E-16
XM_003434933.1		1.61	4.00918E-12
XM_843300.2	Adamts9	1.61	4.07065E-18
XM_531746.3	Ift27	1.61	3.18706E-11
XM_854620.2		1.61	1.88492E-07
XM_541795.3	Bhlhe40	1.61	6.52448E-15
XM_003432029.1		1.61	1.7291E-08
NM_001003248.1		1.61	1.39906E-18
XM_545721.4	Disp1	1.61	1.4244E-12
AY764285.1	Vhl	1.61	9.69972E-11
XM_538741.3	Shb	1.61	4.52802E-17
AY135519.1	Hmgb1	1.61	9.63411E-17
XM_534696.3	Rfc5	1.61	5.93192E-19
XM_846234.2	Tfeb	1.61	8.39255E-07
XM_845597.3	Aif1l	1.61	2.3917E-08
XM_003638786.1	Pqlc1	1.61	2.83558E-14
XM_547003.3	Foxk1	1.61	7.75228E-11
XM_538194.3	Hmgb3	1.61	5.22574E-14
XM_003639281.1	Flot2	1.61	6.28925E-19
XM_849111.2	Cd276	1.61	2.73692E-12
XM_537042.3	Celsr2	1.61	3.45452E-07
XM_546938.4	Sh2b2	1.61	4.72254E-08
XM_863512.2	Midn	1.61	2.39418E-12
XM_003431884.2	Grm8	1.61	1.51933E-09
XM_547091.3	Plk1	1.60	2.62291E-18
XM_544394.3	Nlrc5	1.60	4.045E-09
XM_537787.3	Npdc1	1.60	5.1436E-12
XM_535002.3	Nt5c2	1.60	3.96416E-16
XM_845012.2	Orai2	1.60	2.2997E-09

XM_534457.3	Atp9a	1.60	2.18309E-14
XM_539274.3	Srd5a3	1.60	3.44583E-16
XM_003434735.1		1.60	1.60305E-09
XM_547553.3		1.60	1.79066E-13
XM_848866.2	Rph3al	1.60	1.05298E-08
XM_547713.3	Itpk1	1.60	1.00152E-18
BN000761.1	Arsj	1.60	5.41278E-14
XM_535837.3	Rfc4	1.60	3.20662E-18
XM_847984.2	Chpt1	1.60	2.5367E-14
XM_540756.3	Creb3l1	1.60	4.3645E-08
XM_003433888.2	Loxl1	1.59	4.90253E-15
XM_542187.3	Gadd45b	1.59	7.23158E-19
XM_539597.3	Eif2c4	1.59	1.60218E-11
XM_535470.3	Cops2	1.59	2.54616E-18
XM_532899.3	Cenpa	1.59	3.46406E-13
XM_533516.3	Tle1	1.59	3.2607E-13
XM_543870.3	Foxm1	1.59	2.98726E-12
XM_534988.2	Dnmbp	1.59	6.75435E-12
AY703457.1		1.59	1.25739E-08
XM_003639530.1	Cdc20	1.59	1.00073E-16
XM_548180.2	Hoxb13	1.59	1.28021E-07
XM_003639606.1	Rpia	1.59	7.18688E-15
XM_538324.3	Celsr1	1.59	1.26792E-08
XM_003639333.1		1.59	7.59265E-14
XM_849175.2	Coq5	1.59	5.29207E-12
XM_535499.3	Ccnb2	1.59	1.31452E-12
NM_001048086.1	Psmb9	1.59	7.8858E-11
XM_003433819.1		1.59	8.22036E-11
NM_001114749.1		1.59	4.03593E-11
XM_537829.3	Ciz1	1.59	6.85819E-12
XM_536132.3	F11r	1.59	2.78887E-19
XM_535501.3	Fam81a	1.58	1.13862E-16
XM_540866.3	Capn1	1.58	4.68797E-16
XM_003639120.1	Gdpd3	1.58	1.12992E-08
XM_545497.2	Cobll1	1.58	1.722E-14
XM_540742.3	Ptpmt1	1.58	1.27925E-11
XM_850397.3		1.58	1.69705E-08
XM_542149.3	Plin3	1.58	3.1759E-11
XM_003639086.1	Mafk	1.58	2.4927E-10
XM_003434648.1	Jun	1.58	1.07234E-13
XM_003433621.1		1.58	1.94594E-14
DQ195101.1		1.58	3.33151E-13
XM_003432904.1		1.58	2.11918E-15
XM_003434023.1		1.58	5.33302E-13
XM_537962.3	Asb9	1.58	8.62854E-11
XM_003434507.1		1.58	2.47718E-18
XM_845999.2	Zfhx3	1.58	3.38188E-08
XM_531719.3	Zc3h7b	1.58	8.03227E-15
XM_541489.4	Med25	1.58	1.35883E-11
XM_547284.4	Macc1	1.58	7.00821E-09

XM_844058.2	Prim1	1.58	9.0248E-16
XM_003433399.1		1.58	5.33563E-19
XM_003434816.1		1.58	1.20388E-17
XM_844798.2	Uvrag	1.58	3.15673E-14
XM_846824.2	Pdcd6	1.58	6.9807E-18
XM_546915.3	Clip2	1.58	1.25926E-14
XM_534344.3	Btbd3	1.58	1.01176E-10
XM_549250.1	Elf4	1.57	1.61091E-06
XM_548091.3	Stat5a	1.57	7.22565E-16
XM_846767.2	Tead2	1.57	3.01739E-11
XM_003435571.2	Ctdspl	1.57	2.10006E-10
XM_546939.4	Cux1	1.57	1.19543E-08
XM_534947.3	Ncoa4	1.57	8.01056E-19
XM_532923.3	Dpy30	1.57	1.07424E-15
XM_533994.3	Prcp	1.57	1.888E-14
XM_003431559.1	Lrrc19	1.57	3.12027E-14
XM_541269.3	Rasef	1.57	8.43604E-15
XM_536182.3		1.57	5.19381E-14
XM_539512.2	Dpy19l1	1.57	1.27781E-10
XM_540424.3	Hid1	1.57	1.80148E-05
EU162137.1		1.56	1.01192E-09
XM_544975.3	Pyurf	1.56	9.59555E-17
NM_001270970.1	Pyurf	1.56	1.17269E-16
XM_539197.4	Rhpn1	1.56	6.73076E-12
XM_850179.2	Aldh4a1	1.56	4.23967E-16
XM_844115.1	Inpp5e	1.56	8.96281E-09
NM_001048085.1	Psmb8	1.56	7.10492E-08
AF333433.1		1.56	1.29684E-10
XM_534232.3	Ulk4	1.56	1.10889E-08
XM_543224.3	Esco2	1.56	4.3152E-11
XM_843220.3	Pcyt2	1.56	4.41357E-17
XM_548413.3	Abl1	1.56	7.95185E-13
XM_543844.3	Ptpn6	1.56	8.10134E-09
XM_858566.2		1.56	1.18149E-08
XM_536734.3	Rere	1.56	3.46083E-10
XM_845860.2	Dpy30	1.56	1.51248E-12
XM_848133.2	Rilpl2	1.55	2.19368E-07
XM_543376.3	Hip1r	1.55	8.61848E-18
XM_541589.3	Cic	1.55	7.06572E-09
XM_536657.3	B9d1	1.55	7.90399E-10
DQ489530.1	Map2k1	1.55	1.32746E-17
XM_538707.3	Kif24	1.55	3.59043E-08
AY485421.1	Aspm	1.55	3.71869E-12
XM_536138.3	Ndufs2	1.55	2.07971E-17
XM_848800.3	Evc2	1.55	3.93336E-10
XM_003432855.2	A230050P20Rik	1.55	7.86272E-17
XM_003434841.1	Tmc5	1.55	5.86262E-09
XM_003434617.1	Tmem107	1.55	1.91823E-08
XM_544343.4	Mier3	1.55	1.00411E-06
XM_003435234.2	Traf4	1.55	1.20711E-15

XM_536920.3	Ccdc101	1.55	3.50458E-12
XM_536391.3	Usp54	1.55	2.9094E-10
AF358908.1		1.55	2.82593E-13
XM_541736.4	Mcm2	1.55	2.31888E-16
XM_856413.2	E130309D02Rik	1.55	1.26057E-10
XM_534730.3	Zmat5	1.54	5.35605E-08
XM_537660.3	Lrrc46	1.54	1.22601E-05
XM_852233.2		1.54	9.28232E-08
XM_539928.4	Paxip1	1.54	6.38115E-11
XM_546721.3	Ttll10	1.54	6.03972E-10
XM_003639732.1	Cd320	1.54	1.60165E-11
XM_541418.4	Eps8l1	1.54	1.18311E-16
XM_543812.3	Etv6	1.54	1.2033E-10
AY970669.1	Podxl	1.54	8.80573E-13
XM_534277.3	Mras	1.54	2.97303E-10
XM_541687.4	Arhgap33	1.54	3.06811E-07
XM_847474.3	Smtn	1.54	1.51074E-12
XM_532604.3	Plk3	1.54	1.10951E-10
EU107521.1		1.54	3.51353E-16
XM_546235.4	Dusp1	1.53	2.48465E-16
XM_844113.2	Vps26b	1.53	1.14495E-09
XM_845980.2	Troap	1.53	6.71281E-13
XM_003431781.1	Fam49b	1.53	2.55114E-14
XM_845841.2	Tpx2	1.53	1.63558E-13
XM_532347.2		1.53	1.10993E-07
XM_540818.3	Clcf1	1.53	1.27988E-09
XM_545847.3	Isg20	1.53	4.44222E-08
XM_003640094.1	Col18a1	1.53	6.62241E-12
XM_547356.2	Elf3	1.53	1.98124E-13
XM_548950.3	Mid1ip1	1.53	4.67679E-14
XM_533110.3	Pion	1.53	1.30737E-09
XM_531728.3	Fam83f	1.53	4.09135E-10
NM_001253742.1	Krt19	1.53	6.08299E-15
XM_548877.2	Nhs	1.53	2.59071E-06
XM_003639657.1	Ah k	1.53	5.25691E-13
XM_003432880.1	Prkar2a	1.53	1.5411E-15
XM_843294.2	March8	1.52	1.51323E-08
XM_533192.3	1110051M20Rik	1.52	5.58561E-08
NM_001003245.1	Csf2	1.52	1.25373E-06
XM_003435306.1	Slc46a1	1.52	1.8352E-10
XM_538686.2	Dmrta1	1.52	6.13397E-10
XM_003638992.1		1.52	8.528E-08
XM_533245.3	Vegfb	1.52	3.14723E-06
XM_535726.3	Nfkbiz	1.52	3.63915E-13
XM_846983.2	Ptp4a2	1.52	3.91976E-15
XM_547369.3	Kif14	1.52	1.15822E-07
XM_847977.3	Zfp36l1	1.52	3.55044E-10
XM_003431528.1		1.52	6.25233E-09
XM_541627.3	Pak4	1.52	4.6845E-12
XM_541717.3	Ankrd27	1.52	1.95513E-15

XM_542054.3	Ecsit	1.52	1.43789E-07
NM_001003295.1	Hspb1	1.52	8.24668E-12
XM_003431909.1	Agr2	1.52	1.48241E-11
XM_538256.3	Ctdsp2	1.52	1.7026E-10
XM_003434803.2		1.52	7.615E-10
XM_538389.2	Card10	1.52	6.27324E-13
XM_538855.2		1.52	2.91011E-07
XM_536786.3	Glg1	1.51	4.28921E-13
XM_845706.2	Bicc1	1.51	1.66088E-15
XM_534202.3	Abhd12	1.51	3.85486E-12
XM_533856.3	Lars2	1.51	1.86364E-09
XM_544404.3	Rpgrip1l	1.51	9.98023E-08
XM_536207.4	Tm6sf1	1.51	2.44593E-06
XM_003639919.1	Mtmr3	1.51	3.45635E-15
XM_843736.2	Rufy1	1.51	6.64049E-11
AJ388555.1	Rbm47	1.51	2.69003E-13
XM_847917.2		1.51	1.35191E-06
XM_845415.2	Msrb3	1.51	5.37578E-12
XM_549343.2	Gabra3	1.51	1.32393E-09
XM_003639416.1	Nrm	1.51	3.48369E-12
XM_532169.2	Pkhd1	1.51	2.24563E-14
AF043908.1		1.51	8.56018E-06
XM_549061.3	Kif4	1.51	5.69867E-11
XM_858206.1	Mkl2	1.51	3.57773E-09
XM_003640040.1	Pdcd7	1.51	2.3834E-06
XM_003433423.1	Ccdc157	1.51	1.29511E-06
XM_845131.2	Pcsk7	1.51	1.01358E-14
XM_540746.3	Ddb2	1.51	8.05378E-09
XM_849986.2	Fbxo46	1.51	2.48433E-07
XM_003432833.2	Map1s	1.51	2.19225E-10
XM_848626.2	Tbl1x	1.51	1.37795E-15
XM_548052.3	Plcd3	1.50	4.78736E-10
XM_846161.2	Sytl4	1.50	2.10018E-10
XM_546507.3	Cep164	1.50	1.04561E-12
XM_003434730.1	Thap11	1.50	1.87814E-08
XM_532841.3	Ankrd37	1.50	2.77894E-06
XM_535736.3	Phldb2	1.50	6.33243E-15
XM_540487.4	Gcgr	1.50	3.07311E-12
XM_543049.3	Fam65c	1.50	1.67285E-07
XM_003434473.1	Slit3	1.50	2.98956E-08
XM_543993.3	Trim8	1.50	2.00488E-11
XM_843344.2	Cxxc5	1.50	2.16314E-14
XM_003639547.1	Ttc26	1.50	1.34203E-06
XM_003639182.1	Rabgap1l	1.50	8.35357E-12
XM_846749.2	a60	1.50	2.94728E-08
XM_535735.3	Pvrl3	1.50	2.02264E-09
XM_850316.2	Sipa1l3	1.50	7.36097E-08
XM_849482.1		1.50	7.27409E-06
XM_536468.4	Cd74	1.50	4.86319E-13
XM_003432006.1	Pnp	1.50	9.16846E-15

XM_545906.2	Sh3tc1	1.50	1.5808E-14
XM_542234.3	Maml2	1.50	5.41564E-08
XM_003435661.1		1.50	4.39293E-09
XM_544018.3	Adra2a	1.50	8.10629E-06
XM_548370.4	Agpat2	1.50	6.89812E-12
XM_847753.2	Sfi1	1.50	4.71282E-10
XM_543796.3	H2afj	1.50	6.53832E-06
XM_003432332.1	Vegfb	1.49	7.31886E-11
NM_001145174.1	Car9	1.49	5.20388E-05
XM_003432814.1		1.49	1.36275E-05
XM_844475.2	Vwa5b2	1.49	7.38964E-09
AJ866725.1	Xylt2	1.49	1.48713E-07
XM_536847.3	Tmem120a	1.49	6.45959E-12
XM_534035.3	Prkcdbp	1.49	4.51475E-12
XM_545763.3	Usf1	1.49	3.09174E-14
XM_540813.3	Unc93b1	1.49	3.5567E-11
XM_003639162.1	5730559C18Rik	1.49	8.68573E-09
XM_850960.2	Nfyc	1.49	3.05797E-10
XM_847458.3	Slc39a11	1.49	5.61187E-06
XM_848882.2	E2f4	1.49	2.46549E-12
XM_845495.2	Dgkz	1.49	8.37591E-14
XM_844698.2	Vps8	1.49	1.19995E-14
XM_843488.2	v1	1.49	3.81386E-05
XM_534913.3	Plekhg6	1.49	5.03589E-09
XM_846846.2	Mtch1	1.49	1.01766E-14
XM_542206.3	Stk11	1.49	7.62471E-11
NM_001194977.1	Muc1	1.49	4.59977E-07
XM_003434782.1	Cdc14a	1.49	7.32806E-07
XM_003432803.1		1.49	3.75327E-10
XM_850265.2	Med29	1.49	2.75531E-09
XM_845700.2	Hyal3	1.49	4.04695E-07
XM_003639304.1		1.49	4.86076E-13
XM_843667.3	Zbtb17	1.49	7.19183E-08
XM_003434409.1		1.48	3.02076E-11
XM_542176.3	Mfsd12	1.48	4.73826E-14
XM_003431983.1	Btg1	1.48	1.83838E-14
XM_531632.3	bp2	1.48	1.18129E-14
XM_538386.3	Sh3bp1	1.48	2.13409E-07
XM_545670.3	Wdfy1	1.48	1.99227E-09
XM_855378.2	Gyk	1.48	2.67902E-09
XM_546743.2	Dffb	1.48	2.40666E-06
XM_003638801.1		1.48	5.17216E-10
XM_003435094.1	Homez	1.48	9.70939E-08
XM_546965.3	Ap4m1	1.48	1.33175E-08
XM_547534.3	Mef2d	1.48	5.41054E-06
XM_846973.2	Mad1l1	1.48	2.875E-08
XM_533579.3	Ccdc106	1.48	1.10097E-06
XM_533906.3	Fbxw9	1.48	1.54351E-05
XM_003639917.1	Gltp	1.48	1.66962E-11
XM_850389.2	Fam136a	1.48	1.3853E-08

XM_533065.3	Slc16a1	1.48	9.51487E-11
XM_850056.2	Trappc6a	1.48	9.55318E-07
XM_845155.2	Cldn1	1.48	2.27896E-11
XM_534223.3	Myd88	1.47	3.33023E-08
XM_845822.2	Fanca	1.47	4.4139E-10
XM_844813.2	Aurkb	1.47	2.09487E-12
XM_003432508.2	Lrp11	1.47	7.83174E-06
XM_003433536.1	Mettl7a1	1.47	2.08236E-09
XM_535324.3	Yars	1.47	7.15417E-14
XM_003639341.1	Fam161a	1.47	2.24649E-07
XM_540790.2	Kcnq1	1.47	3.26673E-09
XM_843863.2	Efhd2	1.47	4.53397E-11
XM_003638784.1		1.47	4.63269E-08
XM_547665.3	Arhgap28	1.47	2.55567E-07
HQ637390.1	Lgals9	1.47	9.71074E-07
XM_537673.3	Acsf2	1.47	6.70498E-13
XM_003639829.1	Ppp2r3a	1.47	3.10039E-12
XM_003639841.1	Slc52a3	1.47	7.65405E-05
XM_532902.3	Agbl5	1.47	7.17635E-11
XM_843544.2	Pcsk1	1.47	3.71868E-11
XM_544389.3	Ccdc102a	1.46	1.64485E-10
XM_003433574.1	Gabarapl1	1.46	3.53537E-12
XM_003639051.1		1.46	1.78342E-10
XM_849300.3	Gyg	1.46	6.6888E-09
XM_533102.3	Mll5	1.46	1.22258E-09
XM_849850.3	Trim14	1.46	6.11331E-09
XM_003435514.1		1.46	3.46057E-05
XM_847859.2	Bcar1	1.46	8.91513E-12
XM_549303.3		1.46	3.01454E-08
XM_003639346.1	Zfp36l2	1.46	2.58337E-08
XM_858176.2	Wbp1l	1.46	1.7331E-06
XM_003639067.1		1.46	7.15058E-13
XM_850442.2	Anxa10	1.46	1.04328E-08
XM_847576.2	Zfp428	1.46	4.2433E-07
XM_548201.3	Epn3	1.46	3.65527E-08
XM_847398.2	Spg21	1.46	1.93405E-09
XM_003435226.1	Psmc3ip	1.46	4.25537E-06
XM_541741.3	H1fx	1.46	7.28119E-11
XM_003639101.1	Gadd45a	1.46	4.9415E-13
XM_548179.3	Hoxb8	1.46	8.57869E-06
XM_546930.4	Pom121	1.46	7.24197E-06
XM_845234.2	Smarcd3	1.46	1.38389E-08
XM_003639171.1	Ints3	1.46	4.65892E-10
XM_542177.3	Fzr1	1.46	4.06231E-06
XM_533156.3	Cd59b	1.46	5.3293E-10
FJ159124.1	Igf2	1.46	6.12668E-05
XM_003431725.1		1.45	4.13736E-08
XM_536383.3	Micu1	1.45	9.28545E-12
XM_844761.2	Pomt1	1.45	3.1935E-09
XM_003432704.1	Tmem238	1.45	2.20015E-07

XM_547058.3	Kif22	1.45	1.47428E-10
XM_546204.3	Dbn1	1.45	1.27935E-11
M95495.1	Slc6a6	1.45	4.33866E-10
XM_003639394.1	Clta	1.45	1.08169E-11
XM_532705.3	Fnip2	1.45	6.18599E-11
XM_536387.4	Ttc18	1.45	1.46556E-06
XM_537313.3	Ndc80	1.45	1.30368E-09
XM_533962.3		1.45	3.11325E-13
XM_003639435.1	Lrrc1	1.45	1.87671E-07
XM_543249.3	Pdlim2	1.45	5.00752E-09
XM_844348.2	Irf2bp2	1.45	1.04472E-10
NM_001048101.1	Tapbp	1.45	5.83145E-11
XM_857127.2		1.45	1.62074E-07
XM_547851.3	Syne2	1.45	2.97398E-09
XM_544468.4	Ahdc1	1.45	9.32541E-05
XM_545676.3	Btg2	1.44	2.26289E-07
XM_545648.3	Wnt10a	1.44	2.16433E-07
XM_537369.3	4931414P19Rik	1.44	5.49489E-08
XM_849638.2	Slc25a54	1.44	1.51137E-05
XM_540910.3	Asrgl1	1.44	4.64883E-09
XM_541783.3	Mtmr14	1.44	1.66695E-10
XM_848616.2	Cdh3	1.44	3.17985E-10
XM_844353.2	Mpped2	1.44	2.23735E-12
XM_003434925.1	Ss18	1.44	4.20391E-11
XM_533250.3	a40	1.44	2.04697E-06
AJ286817.1		1.44	3.86091E-07
XM_547142.3	Ubn1	1.44	5.6072E-09
XM_003433936.1		1.44	6.36704E-05
XM_850286.3	Magix	1.44	6.61644E-06
XM_003434024.1	Spp1	1.44	4.54219E-10
XM_533665.3	Shkbp1	1.44	8.9381E-12
M57529.1		1.44	2.23643E-06
XM_843848.2	St3gal4	1.44	9.56518E-12
XM_843702.2	Vwa1	1.44	5.88619E-05
XM_534910.3	Ncapd2	1.44	4.62745E-12
XM_844217.2	Csad	1.44	2.12213E-08
XM_541858.3	Dusp7	1.44	1.6697E-08
XM_539924.3	Galnt11	1.44	1.48123E-10
XM_539600.4	AU040320	1.44	3.64459E-08
XM_536927.4	D430042O09Rik	1.44	5.79339E-06
XM_547642.3	Gata6	1.44	2.79391E-06
XM_544118.3	Ncoa2	1.44	4.51378E-10
XM_003432612.1	Trpm3	1.44	4.50593E-09
XM_532873.3	Klf11	1.44	4.03889E-07
XM_861043.2	Zfp428	1.44	8.10317E-06
XM_847313.2	Pik3r2	1.44	2.45244E-07
XM_003432168.1		1.44	9.89548E-10
XM_546088.3	Disc1	1.44	1.6385E-05
NM_001003253.1	Mal	1.44	3.4875E-12
FJ159123.1	Igf2	1.44	5.41978E-06

XM_849640.2	Nkain1	1.43	1.12265E-08
XM_850020.2	Il22ra1	1.43	8.4518E-06
XM_542917.3	Siglec1	1.43	3.32038E-07
XM_547042.3	Zfp646	1.43	1.17192E-07
XM_532400.3	Rufy3	1.43	2.25989E-07
XM_003432812.1	Twf2	1.43	1.78099E-09
XM_544900.1	Zbtb21	1.43	0.000366916
NM_001115119.1	Gpx1	1.43	1.31335E-11
XM_548346.2	Mrpl41	1.43	3.17624E-08
XM_003433939.1	6030419C18Rik	1.43	2.37023E-07
XM_545360.3	Mboat1	1.43	2.47152E-11
XM_542963.3	E2f1	1.43	1.36546E-06
XM_546756.3	Errfi1	1.43	8.00212E-12
XM_003639450.1	Oplah	1.43	1.09537E-11
XM_847014.2	Mis18a	1.43	1.28696E-06
XM_546823.3	Nudt7	1.43	2.412E-05
XM_850281.2	Pole3	1.43	2.84633E-09
XM_540427.3	Slc16a5	1.43	2.01123E-11
XM_843676.3	Rab18	1.43	1.09834E-10
XM_531630.4	Esyt1	1.43	2.12166E-11
XM_003434861.1		1.43	4.15228E-11
XM_846499.2	Ticrr	1.43	2.23084E-07
XM_849946.2		1.43	2.29586E-06
XM_538425.3	Sh3rf3	1.43	0.000473441
XM_003432515.1		1.43	2.611E-07
XM_540737.2	Ptprj	1.43	2.78333E-09
AB679832.1	Slc9a3r1	1.43	2.12817E-11
XM_849837.2	Sap30l	1.43	8.08857E-05
XM_003432328.1		1.43	4.71588E-09
XM_845065.2	Cited2	1.43	2.26833E-11
NM_001145120.1	Arg2	1.43	2.9614E-11
XM_846750.2		1.42	1.6995E-07
XM_849024.2		1.42	4.88097E-12
XM_849702.2	Ctsz	1.42	5.10608E-12
AY572225.1		1.42	8.01091E-12
XM_536318.3	Iqgap2	1.42	5.78714E-11
XM_003639623.1		1.42	7.86563E-05
XM_536445.3	Pttg1	1.42	1.5405E-08
XM_536720.4	Prdm16	1.42	0.000184243
XM_540418.4		1.42	2.65221E-11
XM_531700.3	Nup50	1.42	1.5904E-09
XM_845993.2	Gtse1	1.42	8.71106E-09
XM_542215.3	Ptbp1	1.42	4.13873E-12
XM_003431726.1		1.42	9.46561E-05
XM_547000.3	Tnrc18	1.42	3.10181E-07
XM_843994.2	Srebf2	1.42	9.31935E-10
XM_850035.2	Rab13	1.42	8.03535E-08
XM_850107.3	R3hdm4	1.42	9.76068E-08
XM_003435231.1		1.42	3.25017E-09
XM_546967.3	Zkscan1	1.42	9.0091E-07

XM_847258.2		1.42	9.91525E-05
XM_535696.3	Enpep	1.42	2.45192E-08
XM_536008.3		1.42	2.56153E-11
XM_537743.4	Spag5	1.42	2.74257E-09
XM_861341.2	Rbmx	1.42	3.0546E-11
XM_540157.3	Sos1	1.42	5.28824E-09
M57532.1		1.42	2.99341E-06
NM_001204929.1	Apitd1	1.42	0.000125279
XM_003434696.1	Zcchc14	1.42	2.78257E-06
XM_543329.4	Ano7	1.42	0.000314256
XM_003432136.1	Sorbs2	1.42	1.48199E-08
XM_537616.3		1.42	0.00013897
XM_546660.3	Rai1	1.42	1.44734E-06
XM_843725.2	Gtf2ird1	1.42	2.73139E-07
XM_547924.3	2310044G17Rik	1.42	7.45765E-06
XM_860971.2	Oat	1.42	1.80253E-11
NM_001011723.1	H2-Ea-ps	1.42	5.45664E-11
XM_853713.2	Rxra	1.42	2.44176E-06
XM_003434102.1	Ccdc127	1.41	3.30303E-07
XM_845276.2	Smarcd2	1.41	5.40408E-11
XM_846629.2	Cnpy3	1.41	4.24288E-10
XM_003434789.1		0.71	2.62992E-07
XM_546652.3	Epn2	0.71	8.62399E-06
XM_546838.3	Ftsjd1	0.71	2.35596E-06
XM_541201.3	Mllt4	0.71	7.67949E-11
XM_003435133.1		0.71	5.03477E-11
XM_535634.3	Coq2	0.71	1.33378E-10
XM_538208.4	Slc10a3	0.71	2.81334E-09
XM_533778.3	Pdhb	0.71	3.75397E-10
XM_848257.3	Igsf5	0.71	7.30767E-07
XM_534645.3	Eif2b1	0.71	8.78893E-09
XM_544546.4	Epha2	0.71	6.08171E-11
XM_003638979.1		0.71	4.3174E-05
AB274721.1	Dhrs4	0.71	2.91958E-11
XM_003638980.1	Ggps1	0.71	3.4525E-07
XM_003434660.1		0.70	8.0255E-08
XM_533524.3	Anxa1	0.70	7.76102E-10
XM_540257.3	Igsf3	0.70	1.90544E-06
XM_547062.3	Kctd13	0.70	0.000114386
XM_548817.2	Fuom	0.70	1.64643E-05
XM_538246.3	Stac3	0.70	4.26443E-05
XM_536727.3	Acot7	0.70	1.67543E-11
AY911512.1	Hspa4	0.70	2.80725E-11
XM_846462.1	Specc1	0.70	2.25912E-08
XM_532737.3	Tpk1	0.70	2.19857E-09
XM_537207.3	Tiprl	0.70	2.14681E-07
XM_541147.3	Ppil4	0.70	1.65056E-07
XM_537617.3	Ccdc43	0.70	4.83432E-08
XM_548277.3	Crlf3	0.70	1.23892E-07
XM_538538.3	Pcyox1	0.70	2.1653E-10

XM_862584.2	D jb4	0.70	3.30483E-07
XM_540914.3	Fads1	0.70	1.28009E-05
XM_542692.3	Fbxl2	0.70	6.86742E-05
XM_532920.3	Wdr43	0.70	2.79241E-10
AY057077.1	Tpmt	0.70	8.41734E-07
XM_003434843.1	Itpripl2	0.70	3.65579E-05
XM_845085.2	Tmem5	0.70	3.67193E-06
XM_536728.3	Plekhg5	0.70	3.53642E-05
XM_540220.2	Mogs	0.70	9.80086E-11
XM_535019.3	A630007B06Rik	0.70	9.23854E-07
XM_847167.2	Tmem59l	0.70	2.19432E-08
XM_543330.2	Farp2	0.70	5.21591E-06
XM_535987.3		0.70	8.01492E-07
XM_535330.3	Ti gl1	0.70	4.47903E-11
XM_849137.2	Ap1s2	0.70	1.48266E-05
XM_534397.3	Myh7b	0.70	2.29572E-05
BN000767.1	Arsk	0.70	1.12454E-07
XM_850655.2	Hsp90ab1	0.70	8.9697E-05
XM_545596.3	Trak2	0.70	2.9771E-07
XM_535130.3	1110037F02Rik	0.70	1.88633E-11
XM_541861.3	Parp3	0.70	3.74087E-10
NM_001195695.1	Gm166	0.70	0.000524958
HQ189123.1	Dapk1	0.70	4.28304E-09
XM_003435112.1	Khnyn	0.70	5.11516E-08
XM_849699.2	Nr2c1	0.70	5.13664E-08
XM_543458.3	Tpst2	0.70	9.42197E-05
XM_536001.3	Osgepl1	0.70	4.30591E-06
XM_534923.3	Fkbp4	0.70	1.06483E-10
XM_003434138.1	B3galnt1	0.70	3.93996E-07
XM_860702.2	Lrif1	0.70	3.0179E-07
XM_536703.3	0610037L13Rik	0.70	1.93449E-09
XM_546094.3	Ttc13	0.70	4.58753E-10
XM_853737.2		0.70	2.89496E-07
XM_003432050.1	Bpgm	0.70	2.01889E-05
XM_844270.2	Wbp2	0.70	9.38344E-12
XM_536131.3	Copa	0.70	7.45865E-12
XM_003639860.1	Srsf6	0.70	1.06448E-11
XM_532358.3	Kifc2	0.70	6.45176E-09
XM_535140.3	Cul2	0.70	1.06814E-09
XM_536077.3	Tuba4a	0.70	6.25069E-10
XM_003639501.1	Yrdc	0.70	5.54275E-12
XM_003639600.1	Polr1b	0.70	1.57411E-10
XM_534632.3	Golga3	0.70	1.36611E-10
XM_850688.3	Rchy1	0.70	1.65632E-06
XM_846893.2	Sugt1	0.70	3.71806E-10
XM_540195.3	Tmem150a	0.70	1.22396E-06
XM_546029.3	Arrdc3	0.70	7.01695E-08
XM_533007.3	Smyd5	0.70	1.13342E-11
XM_537245.3	Cct3	0.70	8.03211E-11
NM_001136563.1	Pip5k1a	0.70	1.22975E-11

XM_543322.4		0.70	1.13661E-09
XM_548490.4	Creb3l2	0.70	7.01066E-07
XM_003435250.1	Nr1d1	0.70	0.000179524
XM_539073.4	Lace1	0.70	1.12036E-05
XM_545056.4	Arl13b	0.70	5.77591E-07
XM_847787.2	Mrm1	0.70	1.1705E-06
XM_534129.1	Lacc1	0.70	1.20383E-06
XM_535120.3	Fam82b	0.70	2.95427E-07
XM_540010.3	Cnot7	0.70	4.33826E-12
XM_531854.4	Gfpt1	0.70	1.79126E-11
XM_003434244.1	Phospho2	0.70	6.80653E-07
XM_535576.3	Cct8	0.69	2.11206E-10
XM_848402.2	Nol11	0.69	3.90577E-09
XM_538376.3	Maff	0.69	2.29475E-06
XM_849009.3	Ccnc	0.69	1.45741E-08
XM_533330.3	Slc35f5	0.69	3.6276E-11
XM_544859.3	Urb1	0.69	1.79102E-09
XM_546422.3	Ccdc15	0.69	1.19149E-07
XM_536386.3	Ecd	0.69	1.51582E-09
XM_003435623.1	Tceal8	0.69	3.44923E-05
NM_001131047.1	Ak1	0.69	1.25977E-11
XM_546694.3	Pars2	0.69	2.28747E-05
XM_003433350.1	Psmd1	0.69	2.66245E-12
XM_843406.2	Ankrd29	0.69	0.000143568
XM_536367.3	Herc4	0.69	1.83488E-08
XM_003432936.1		0.69	2.83443E-05
XM_541278.3	Nmrk1	0.69	1.2036E-05
XM_535606.3	G3bp2	0.69	8.32222E-12
XM_850400.2	Fxyd5	0.69	3.93242E-09
XM_003435053.1		0.69	7.94562E-06
XM_003433681.1		0.69	2.58618E-07
XM_534803.3	Larp4	0.69	1.18214E-10
XM_843527.2	Pcmtd1	0.69	6.07032E-09
XM_844064.2	Vash2	0.69	6.44385E-11
XM_844122.2	Nif3l1	0.69	3.77562E-07
XM_859506.2		0.69	5.51215E-06
XM_003431963.1	Tmem69	0.69	4.8538E-07
XM_542641.3	Gpr180	0.69	2.6497E-08
XM_846175.1	Elovl7	0.69	1.58004E-07
XM_537414.3	Brms1l	0.69	3.0543E-05
XM_003640277.1	Spin2	0.69	1.26302E-05
AJ388545.1		0.69	4.2837E-06
XM_535226.3	Yipf5	0.69	6.03746E-12
XM_003435018.1		0.69	5.56432E-13
XM_003639104.1	Usp31	0.69	2.37719E-06
XM_544990.3	Rap1gds1	0.69	8.205E-09
XM_534998.3	Pprc1	0.69	3.21342E-12
XM_537982.3		0.69	1.2725E-05
XM_003639987.1	Noc3l	0.69	1.53091E-10
XM_535692.3	Ccdc109b	0.69	8.51764E-06

XM_532552.3	Utp11l	0.69	2.29118E-10
XM_003433895.1		0.69	5.17432E-05
XM_844363.1	BC027231	0.69	1.31512E-08
XM_535212.3	Wdr55	0.69	1.61938E-10
XM_535511.3	Csnk1g1	0.69	1.42197E-07
XM_003435660.1	C1galt1c1	0.69	3.35613E-09
NM_001003270.1	Ssr1	0.69	4.17694E-13
XM_003640185.1	Tmem237	0.69	8.02454E-09
XM_850987.2		0.69	3.12965E-11
XM_003431573.1	Rgp1	0.69	1.56025E-09
XM_859235.2		0.69	4.27885E-08
XM_844464.2	Arhgef16	0.69	3.20731E-12
XM_545093.1	Zbed3	0.69	2.85874E-08
XM_543228.3	Ptk2b	0.69	1.12526E-06
XM_541532.4		0.69	1.4352E-07
XM_003434301.1	Glrx2	0.69	1.49272E-06
XM_544913.3	Pdxk	0.68	2.24971E-09
XM_003432138.1	4933411K20Rik	0.68	2.36578E-07
XM_003639939.1	Cops7a	0.68	1.05059E-11
XM_543466.3	Kremen1	0.68	2.13976E-06
XM_538210.4	Vbp1	0.68	3.98236E-09
XM_547338.3	Lrrc40	0.68	1.45754E-08
XM_533348.3	Orc4	0.68	3.33481E-10
XM_539884.3	Slc37a3	0.68	1.42537E-09
XM_850155.3		0.68	1.58506E-06
XM_863149.2	Rab34	0.68	7.63288E-13
XM_843910.1	Strip2	0.68	2.79688E-05
XM_537828.3	Golga2	0.68	1.04517E-13
XM_535174.3	Stam	0.68	3.79603E-10
XM_539736.3	Ikbip	0.68	1.23577E-09
NM_001252367.1		0.68	3.81337E-09
XM_845760.2	Prps1	0.68	7.20381E-11
XM_538234.2	Spryd4	0.68	2.31337E-06
XM_534000.2	Ints4	0.68	1.77286E-11
XM_845924.2	Ehd4	0.68	1.269E-10
XM_538704.3	Nol6	0.68	1.7297E-06
XM_850426.2		0.68	9.97448E-09
XM_536965.3	Rrn3	0.68	3.85267E-11
XM_537306.3	Usp14	0.68	9.21386E-07
XM_537730.3	Psmd11	0.68	1.30615E-13
XM_541124.2	D10Bwg1379e	0.68	6.89899E-07
XM_543139.3	Pds5b	0.68	2.9663E-08
XM_543444.3	Coro1c	0.68	5.56336E-06
XM_843195.2	Psph	0.68	2.47298E-13
XM_535952.3	Ssb	0.68	1.89785E-11
AF217203.1	Sgsh	0.68	1.66561E-08
XM_545821.3	Chsy1	0.68	1.48548E-07
XM_537624.3	Dusp3	0.68	5.07553E-08
XM_003431455.1	Tbc1d15	0.68	5.94051E-12
XM_843772.2	Ddx1	0.67	1.03998E-12

NM_001194952.1	Plau	0.67	1.78189E-11
XM_845257.2	Slc30a5	0.67	5.27532E-14
XM_533399.3	Mex3c	0.67	8.92913E-13
XM_003432488.1		0.67	1.71973E-13
XM_535620.3	Mrpl1	0.67	6.70522E-10
AY871202.1	Zdhhc8	0.67	1.26066E-09
XM_845336.2	Kdelr2	0.67	4.38223E-14
XM_003639002.1	Ndst2	0.67	3.18495E-08
EF102104.1	Birc3	0.67	5.78978E-10
XM_532990.3	Mrpl19	0.67	1.08935E-11
XM_844153.2	Rnf14	0.67	9.9632E-13
XM_545985.4	Dgkq	0.67	5.18337E-11
XM_003638766.1	Tmem181a	0.67	2.43538E-08
XM_546341.3	Osmr	0.67	4.72537E-11
XM_863433.2	Dicer1	0.67	6.00959E-12
XM_534986.3	Cox15	0.67	1.75333E-11
XM_003431866.1	Ispd	0.67	9.8901E-11
XM_847360.2	Xpo5	0.67	2.57E-13
XM_548376.4	cc2	0.67	6.44671E-09
XM_003432942.1		0.67	2.8043E-08
XM_851960.2	Sec23b	0.67	1.08798E-13
XM_855513.2	Mon2	0.67	2.75088E-14
XM_848223.2		0.67	2.20353E-06
XM_003433374.1		0.67	1.27458E-10
XM_534014.3		0.67	1.34642E-08
XM_855340.2	Tubb6	0.67	1.84947E-14
XM_534949.3	Zfand4	0.67	5.24167E-10
XM_536504.3	Brix1	0.67	3.85107E-11
XM_844156.2	Styxl1	0.67	3.95488E-09
XM_532927.3	Ttc27	0.67	1.12973E-14
XM_846930.1	Chst12	0.67	3.40473E-06
M55251.1	Clu	0.67	2.40788E-13
XM_535636.3	Fam175a	0.67	6.18636E-07
XM_003433474.1	Mrpl51	0.67	2.66706E-09
XM_538283.3	Ptprb	0.67	4.4055E-11
XM_003431494.1	Sept10	0.67	8.21501E-14
XM_539020.3	Tpbg	0.67	7.03072E-15
XM_539575.3	Smap2	0.67	4.84245E-12
XM_534871.3	Pyroxd1	0.67	6.86769E-12
XM_546476.3	Tbcel	0.67	2.66465E-06
XM_843631.2	March7	0.67	2.81477E-13
XM_847897.2	Arl1	0.67	1.76532E-14
XM_003640213.1	Igsf8	0.67	2.33797E-09
XM_539997.3	Eri1	0.67	4.39849E-07
XM_854327.2		0.67	1.45133E-06
XM_846262.2	AI413582	0.67	1.89459E-09
XM_003433672.1		0.67	2.42352E-08
XM_542640.3	Tgds	0.67	2.6585E-10
XM_542543.3	Lin7c	0.67	4.19612E-06
XM_541950.3	Glt25d1	0.67	3.70077E-13

XM_844200.2		0.67	1.64941E-11
XM_539209.3		0.66	9.65481E-09
XM_541050.3	Zfp516	0.66	1.74399E-06
XM_542135.2		0.66	8.67178E-09
XM_845987.2	Taf1a	0.66	1.05603E-07
XM_539730.3	Elk3	0.66	1.41943E-09
XM_545575.3	Stk17b	0.66	1.0083E-07
XM_533461.3	Serac1	0.66	6.28085E-10
XM_545701.3	Rgs2	0.66	9.41428E-10
XM_003434290.1		0.66	8.34792E-13
XM_849592.2	Ptgr2	0.66	9.30575E-09
XM_863517.2	Eif5	0.66	3.24151E-15
XM_846483.2	Spata5l1	0.66	2.241E-09
XM_543098.3	Helz2	0.66	6.93697E-12
XM_542770.3	Satb1	0.66	1.39058E-06
XM_843780.2	Tmem194b	0.66	3.60087E-08
XM_534841.4	Cpne8	0.66	1.94723E-07
XM_544569.3	Fbxo6	0.66	4.98692E-14
XM_003639328.1	Prorsd1	0.66	2.14423E-10
XM_849897.3	Ostm1	0.66	4.95296E-12
NM_001194969.1	Hmox1	0.66	1.13625E-05
XM_539054.3	Usp45	0.66	6.20326E-06
XM_845934.2	Fam174a	0.66	1.31367E-07
XM_537589.3	Smurf2	0.66	1.12998E-11
XM_532390.3	Uba6	0.66	1.89841E-14
XM_532546.3	Mfsd2a	0.66	5.25392E-11
XM_539631.3	Mmachc	0.66	4.83506E-06
DQ403086.1		0.66	2.44005E-14
XM_844774.2	Uap1	0.66	1.08844E-13
XM_533390.3	Lman1	0.66	4.86083E-15
XM_532911.3	Nrbp1	0.66	8.78542E-17
XM_844258.2	Mphosph10	0.66	5.30848E-12
XM_003432675.1	Zfp74	0.65	1.57985E-07
XM_531646.3	Mars	0.65	1.92696E-14
XM_003432736.1		0.65	1.07174E-10
XM_548003.3	Prx	0.65	3.50532E-10
XM_003433546.1		0.65	7.13834E-07
XM_544259.3	Echdc3	0.65	7.85632E-08
XM_532846.3	Ccdc111	0.65	9.90832E-09
XM_539885.3	Jhdm1d	0.65	8.11517E-10
XM_858957.2	Frmd6	0.65	1.08492E-07
XM_536289.3	Pam	0.65	1.01446E-14
XM_533083.3	Rala	0.65	8.84572E-15
XM_532602.3	Urod	0.65	1.42774E-15
XM_003435124.1		0.65	9.05041E-07
XM_535006.3	Col17a1	0.65	3.35804E-15
XM_538678.3	Focad	0.65	1.72951E-13
XM_846215.2	Adprm	0.65	4.84092E-09
XM_844375.2	Eprs	0.65	1.40728E-16
XM_535437.3	Exd1	0.65	6.63838E-06

XM_545216.3		0.65	5.623E-16
XM_540937.3	a15	0.65	7.93923E-15
XM_847665.2	A730098P11Rik	0.65	4.35071E-16
XM_535275.3	Hexb	0.65	1.16998E-06
XM_533770.4	Magi1	0.65	1.26917E-07
XM_536477.3	Pelo	0.65	3.50737E-14
XM_539521.3		0.65	5.19023E-12
XM_844325.2	Wdr37	0.65	5.02854E-11
XM_545565.3	Wdr75	0.65	5.77524E-16
XM_535239.3	Il6st	0.65	2.71391E-13
XM_844117.2	Wnt7a	0.65	2.52077E-16
XM_003640125.1	1700021K19Rik	0.64	1.51384E-10
XM_849749.2	St3gal5	0.64	5.19489E-09
XM_534086.3	Spty2d1	0.64	1.07613E-11
XM_003639587.1	Hk2	0.64	2.91803E-16
XM_534690.3	Tpcn1	0.64	1.4145E-08
XM_532060.3	Tubb5	0.64	1.2509E-16
XM_533303.3	Anxa5	0.64	1.04261E-17
XM_532310.2	Sybu	0.64	1.06932E-07
XM_847692.2	Fam214b	0.64	9.31421E-11
AY044905.1	Ptgs2	0.64	0.000153125
XM_533025.3	Sec22b	0.64	1.07896E-14
XM_854115.2	Alg3	0.64	2.0437E-13
XM_003435547.1	Zfp185	0.64	6.87326E-07
XM_546032.3	Cetn3	0.64	7.32244E-17
XM_003433165.1	Ano10	0.64	9.97848E-12
XM_846536.2	Wdyhv1	0.64	1.31543E-10
XM_846019.2	Cep170	0.64	5.19799E-14
XM_535684.3	Sgms2	0.64	6.18282E-14
DQ353847.1		0.64	1.16358E-06
XM_003639608.1	Rab11fip5	0.64	2.50034E-12
XM_003435116.1		0.64	8.46616E-16
XM_003431975.2	Zfp219	0.64	2.86406E-07
XM_843573.2	Dars	0.64	3.48503E-08
XM_003639595.1	Fahd2a	0.64	2.8246E-11
XM_853776.2	Siae	0.64	1.01139E-07
XM_843638.3		0.64	5.06687E-10
XM_533645.3		0.64	1.41017E-11
XM_845446.2	Ascc1	0.64	4.96276E-09
XM_003638878.1	Kif5b	0.64	2.84565E-17
XM_535037.3	Sec23ip	0.64	1.94953E-17
XM_535630.3	Sec31a	0.64	1.42604E-18
XM_535778.2		0.64	1.10134E-10
AB674458.1		0.64	5.11116E-15
AF314533.1	Il13ra2	0.64	8.77878E-07
XM_535702.3	Sec24d	0.63	1.37249E-18
XM_537485.3	Eif2s1	0.63	6.47688E-15
XM_003433261.1		0.63	4.56853E-11
XM_540899.3	Wdr74	0.63	3.14906E-15
XM_540360.3		0.63	6.43795E-10

XM_535044.3	Htra1	0.63	3.90327E-12
XM_549180.4	gk	0.63	3.74164E-15
XM_534111.3	Spryd7	0.63	3.11705E-11
XM_536009.3		0.63	6.75269E-11
XM_003639108.1	Fam86	0.63	5.37328E-14
XM_546726.3	Plekhn1	0.63	7.71529E-15
XM_546591.2	Chrnb1	0.63	7.61958E-10
XM_003433615.1	Taf5	0.63	1.01527E-09
XM_533732.3	Plxnd1	0.63	1.38837E-09
XM_850347.2		0.63	2.62796E-06
XM_537419.3	Trappc6b	0.63	1.81054E-07
XM_845797.2	Fam160a2	0.63	2.56256E-11
XM_845480.1	Mars2	0.63	2.89638E-09
XM_003639977.1	Mansc1	0.63	4.65205E-12
XM_847165.2	Ccdc88a	0.63	5.52989E-09
XM_533444.3	Ginm1	0.63	1.44926E-15
AB200288.1	F3	0.63	7.76821E-14
AY455801.1		0.63	1.06492E-13
XM_843276.2	Esd	0.63	3.39211E-16
XM_539117.3	Lrp12	0.63	1.47094E-08
XM_542644.3	Uggt2	0.63	1.69415E-11
XM_531785.3	Txndc9	0.62	1.87522E-15
XM_536302.3	Rasa1	0.62	4.71537E-18
XM_850372.2	6030458C11Rik	0.62	5.38599E-11
XM_003433320.1	Pid1	0.62	8.95122E-08
XM_536015.3		0.62	3.98099E-14
XM_003639826.1	Uba5	0.62	1.5485E-17
XM_003640209.1	Lyplal1	0.62	9.3673E-13
XM_536876.3	Baiap2l1	0.62	3.98107E-18
XM_533542.3	Cdc37l1	0.62	7.11295E-13
NM_001252581.1	Syngr2	0.62	5.04826E-19
XM_536530.3	Aplp2	0.62	1.41006E-19
XM_531681.3		0.62	1.15565E-09
AY563546.1		0.62	1.33757E-11
XM_540214.3	Sema4f	0.62	1.35872E-15
XM_845301.2	Sash1	0.62	1.25896E-07
XM_850121.2	Tube1	0.62	1.42981E-13
XM_843396.2	Cog3	0.62	4.56737E-16
XM_003640023.1	Lcmt2	0.62	7.63087E-10
XM_549134.3	Srpx2	0.62	4.12184E-14
XM_003431509.1		0.62	1.40105E-17
XM_003638848.1		0.62	1.41028E-09
XM_843295.2	Nqo2	0.62	5.03626E-17
XM_535624.3	Anxa3	0.62	2.85687E-12
XM_533459.3	Tfb1m	0.62	9.26841E-08
XM_856982.2	Eif5a2	0.62	6.22309E-08
NM_001003291.1	Icam1	0.62	1.32763E-17
XM_003638770.1	L3mbtl3	0.62	1.4876E-06
XM_859070.2	Serpine2	0.61	3.05211E-11
XM_544107.1	Rrs1	0.61	3.08525E-13

XM_537963.3	Pir	0.61	9.87039E-17
XM_003638949.1		0.61	6.32493E-10
XM_536028.3	Orc2	0.61	1.35433E-17
XM_534495.3	Spg20	0.61	4.05506E-17
XM_535944.3	Stk39	0.61	5.54485E-11
XM_003638825.1	Ppp1r13l	0.61	9.35508E-16
XM_856221.2	Rpe	0.61	6.65649E-13
XM_536390.3		0.61	1.65284E-16
XM_862709.2		0.61	2.9636E-13
XM_539015.3	Elovl4	0.61	1.59517E-08
XM_846454.2	Nradd	0.61	4.519E-12
XM_537584.3	Psmd12	0.61	1.99778E-17
XM_539700.3	Mettl25	0.61	1.75866E-07
XM_531737.3	Kdelr3	0.61	2.33035E-19
XM_536047.3	Idh1	0.61	7.7967E-18
XM_532542.3	Exo5	0.61	4.75125E-17
AF016649.2		0.61	1.88653E-20
DQ195097.1		0.61	4.41659E-10
AY266682.1		0.61	2.01245E-13
XM_003435004.1		0.61	4.04334E-14
XM_544130.3	Jph1	0.60	2.23631E-07
XM_531810.3	Socs5	0.60	2.51649E-09
XM_543638.3	Spryd3	0.60	1.62932E-13
NM_001003198.1	Pla2g7	0.60	2.37856E-17
NM_001003272.1	Srp54a	0.60	1.02422E-20
XM_843485.3	Stk25	0.60	1.89251E-19
XM_536702.3	Magoh	0.60	1.48016E-18
XM_850002.2	Tspan12	0.60	5.55964E-21
XM_544713.3	Lactb	0.60	2.20817E-11
XM_544918.4	Icosl	0.60	6.28868E-08
XM_537404.3	Strn3	0.60	8.45194E-16
XM_003433223.1	Fastkd5	0.60	1.54421E-08
L31625.1		0.60	1.04008E-20
XM_535950.2	Fastkd1	0.60	3.17612E-13
XM_003639212.1	Klc1	0.60	5.39399E-18
XM_856985.2	Dixdc1	0.60	5.17187E-12
XM_846683.2	1190005I06Rik	0.60	9.06604E-18
XM_541015.3	Mgat5	0.60	1.89035E-13
XM_003639383.1	Acer2	0.60	2.39746E-08
XM_003639237.1		0.60	5.43244E-13
XM_546030.3	Lysmd3	0.60	1.27148E-11
XM_846856.2	Maged2	0.60	7.51629E-23
XM_848630.2	Ap2b1	0.60	3.78967E-23
XM_003433114.1		0.60	3.32004E-15
XM_538271.3	Irak3	0.60	1.14804E-10
XM_844784.2	Etv5	0.59	6.58064E-08
XM_540364.2	Inhba	0.59	1.04712E-07
XM_003434594.1		0.59	2.78094E-15
XM_540366.3	5033411D12Rik	0.59	1.46096E-08
XM_846169.2	Eif5b	0.59	1.40745E-22

XM_536071.3	Rnf25	0.59	7.84114E-18
AB793315.1		0.59	5.93212E-11
XM_843280.2	Serpinb6a	0.59	5.20766E-24
XM_003433926.1		0.59	3.13778E-10
AY965264.1	Igf2r	0.59	8.2635E-20
XM_542723.3	Entpd3	0.59	9.98198E-22
XM_546317.3	Fbxo38	0.59	3.76208E-19
XM_849493.2		0.59	2.10987E-23
XM_544620.3	Rpusd2	0.58	4.89988E-12
XM_533392.3	Malt1	0.58	2.10285E-14
XM_003639661.1	1600016N20Rik	0.58	6.26658E-09
XM_543000.3	Lpin3	0.58	7.70718E-09
FJ591132.1		0.58	1.45607E-10
XM_538416.3	Ckap4	0.58	3.57017E-23
XM_003434571.1		0.58	9.61302E-26
XM_543660.2	Krt80	0.58	1.94299E-16
XM_539402.3	Crot	0.58	9.7835E-22
DQ975208.1	Ceacam1	0.58	1.28503E-18
FJ870766.1		0.58	7.89612E-10
XM_003432798.1	Gnl3	0.58	1.12869E-21
XM_003435279.1		0.58	7.95796E-09
XM_846177.2	9930012K11Rik	0.58	8.34345E-14
XM_541510.2	Fgf21	0.58	2.51134E-17
XM_003639527.1	Hpdl	0.58	2.07656E-12
XM_845344.2	Sec13	0.58	2.58452E-26
XM_536768.3	Gcsh	0.58	2.50754E-16
XM_539472.3	Gpnmb	0.58	4.72772E-22
XM_535225.3	Nr3c1	0.57	6.29208E-15
XM_844079.2	Morn1	0.57	3.55904E-11
XM_532994.3	Dqx1	0.57	2.72892E-20
XM_546405.3	Ets1	0.57	1.62556E-16
XM_541920.2	Cilp2	0.57	6.74009E-10
XM_547116.3	Pdxdc1	0.57	1.07044E-27
XM_543987.3	Gbf1	0.57	2.51557E-22
XM_863036.2	Fbxl4	0.57	8.89325E-21
XM_547394.3	Diexf	0.57	6.68329E-22
XM_544610.3	Thbs1	0.57	2.3398E-21
XM_537356.3	Golga5	0.57	4.62582E-25
XM_547668.3	Twsg1	0.57	2.07137E-12
XM_546666.3	Map2k3	0.57	2.6938E-21
XM_532421.3	Copg2	0.57	6.30325E-23
XM_003433614.1		0.57	6.8333E-20
XM_547836.3		0.57	4.22533E-20
XM_533335.3	Zranb3	0.57	1.73316E-22
XM_547419.3	Qsox1	0.57	6.50903E-25
M68524.1		0.56	4.30371E-16
XM_846466.2	Glrx	0.56	1.98944E-10
XM_003433773.2		0.56	3.61102E-13
XM_536552.3	Arcn1	0.56	1.52451E-21
XM_536055.3	Lancl1	0.56	4.48557E-25

XM_541778.3	Rpusd3	0.56	2.30899E-18
XM_532248.3	Aim1	0.56	3.51144E-14
XM_846481.2	Itpa	0.56	2.24526E-22
XM_844242.2	Calu	0.56	5.63218E-29
XM_535882.3	Eef1e1	0.56	1.08119E-19
XM_541933.3	Klhl26	0.56	4.03274E-15
NM_001197170.1	Col9a2	0.56	4.5498E-18
XM_003434948.1	Dsc3	0.56	1.35346E-16
XM_546411.3	Srpr	0.55	1.61522E-30
XM_003433853.1	Sfn	0.55	2.78434E-28
XM_544628.3		0.55	1.06062E-25
XM_003432416.1	1810055G02Rik	0.55	3.52998E-18
XM_844835.2	Mgarp	0.55	2.48229E-13
XM_849226.3	Rgs11	0.55	3.61226E-14
XM_003639541.1		0.55	4.19748E-23
XM_003431510.1	Mterfd3	0.55	5.03748E-13
NM_001197096.1	Ppp3ca	0.55	6.7754E-21
XM_543235.3	Kctd9	0.55	3.82389E-15
XM_533723.3	Copg1	0.55	3.56246E-31
XM_846034.2	Mrpl17	0.55	6.40741E-27
XM_536545.3	Sorl1	0.55	2.15576E-18
XM_538640.3	Tgfbi	0.55	8.74371E-13
XM_003432793.1		0.55	1.6733E-11
XM_544994.3	Trmt10a	0.55	7.36883E-18
XM_848852.2	Wsb1	0.55	8.28098E-29
XM_534051.3	Wee1	0.55	9.34742E-29
XM_546083.3	Kcnk1	0.55	3.84935E-20
AY292464.1	LOC101056437	0.55	5.57158E-18
XM_547684.3	Spire1	0.54	2.52809E-11
XM_843361.2	Rnf19a	0.54	1.01082E-30
XM_544163.3	Cpne3	0.54	1.18282E-19
XM_545846.3	Aen	0.54	5.82452E-26
XM_848021.2	Ppif	0.54	1.51205E-25
XM_535989.3		0.54	5.05981E-10
XM_847205.2	Sh3gl3	0.54	4.53735E-17
XM_845057.2	Ralgps2	0.54	3.43717E-20
XM_003434288.1	Hecw2	0.54	2.11992E-09
XM_539996.3	Ppp1r3b	0.54	3.69428E-17
XM_544097.3	Chd7	0.54	6.1801E-16
XM_843457.2	Tbc1d23	0.54	1.79334E-27
XM_537156.3	Lamc1	0.53	6.49108E-34
XM_003435631.1	Morf4l2	0.53	1.63469E-33
EU263364.1		0.53	6.05959E-32
XM_843599.2	Clic3	0.53	1.55028E-26
XM_547805.3	Atp5s	0.53	1.53786E-13
XM_534148.3	Lmo7	0.53	1.76558E-25
NM_001197095.1	Serpine1	0.53	1.74178E-25
XM_003435633.1	Morf4l2	0.53	3.10527E-33
XM_535418.3	Aven	0.53	1.55407E-22
XM_535925.4	Tanc1	0.53	3.66816E-10

XM_845599.2	Rgs17	0.53	5.09619E-14
XM_537128.3	Ddx59	0.53	3.16514E-29
XM_535077.2	Tgs1	0.53	1.50957E-19
XM_847034.2	Gpr155	0.53	2.28239E-12
XM_848855.2	Bnc2	0.53	2.33707E-10
XM_003435324.1		0.53	2.23227E-25
XM_548004.3	Jag2	0.53	2.16317E-25
XM_844594.2	Ppap2a	0.53	7.77749E-22
XM_003435130.1	Nin	0.53	1.51851E-12
XM_003639810.1	Tex30	0.53	4.15022E-19
XM_854951.2	Klf7	0.53	3.32104E-22
XM_003435145.1	Wdr89	0.52	8.39747E-13
XM_533382.3	Serpinb5	0.52	8.25509E-23
XM_538243.3	b2	0.52	1.79747E-14
XM_853765.2	Myof	0.52	7.87804E-31
XM_849657.2	Creb3	0.52	1.40008E-32
XM_539915.3	Atg9b	0.52	3.22371E-22
XM_539002.3	Col12a1	0.52	1.16989E-19
XM_537061.4	Dpyd	0.52	1.54937E-32
XM_003432569.1		0.52	1.5195E-31
XM_845324.3	Lix1l	0.52	1.07808E-17
XM_538044.4	Maged1	0.52	2.27204E-31
XM_540955.3		0.52	1.61952E-20
XM_539995.3	Dusp4	0.52	7.5299E-24
XM_535548.4	Ube2q2	0.52	1.51612E-11
XM_532322.3	Zhx1	0.52	7.45031E-16
JX144398.1		0.52	9.3487E-22
XM_003434371.2	Fgfrl1	0.52	5.27639E-19
XM_538928.3	Cul7	0.52	2.73543E-35
XM_538748.3	Tbc1d2	0.51	1.62447E-27
XM_534237.3	Tgfbr2	0.51	3.25353E-38
XM_847945.2	Gosr2	0.51	2.90116E-22
XM_534510.3	Fry	0.51	3.173E-17
XM_539524.3	Dock4	0.51	4.70609E-19
XM_532216.3	Pgm3	0.51	9.51092E-37
XM_533434.3	Phactr2	0.51	6.86241E-16
XM_533073.3	Egfr	0.51	8.28653E-22
XM_003434708.1		0.51	4.42287E-13
XM_003640141.1	Zmat3	0.51	4.8357E-16
XM_542831.3		0.51	5.78723E-23
XM_850178.2	R set2a	0.50	5.16056E-30
XM_003433220.1	Smox	0.50	7.29245E-17
XM_545329.3	Dsp	0.50	1.71773E-37
XM_847039.3	Cmtm6	0.50	2.54547E-17
AF143503.1		0.50	4.68902E-14
XM_846330.2	Nedd4	0.50	3.56913E-23
XM_531763.3	Aldh1l2	0.50	9.63588E-14
XM_003431715.1	Dll1	0.50	6.37559E-11
AY745241.1	Lifr	0.50	6.39655E-27
XM_003433595.1	Sorbs1	0.50	3.70403E-11

XM_548021.3	Wipi1	0.50	1.05698E-19
NM_001122778.1	Txnrd3	0.50	1.78985E-16
XM_532929.3	Fam98a	0.50	1.78021E-38
XM_542452.3	Smpd1	0.50	1.53818E-21
XM_854989.2		0.50	1.1507E-12
XM_535505.3	Tln2	0.50	2.7097E-24
XM_538032.3	Praf2	0.49	7.35241E-38
XM_532995.3	Pcgf1	0.49	1.82593E-34
XM_003432767.2	Fbln2	0.49	6.03103E-17
XM_540219.3	Lbx2	0.49	9.00061E-24
XM_542919.3	Slc4a11	0.49	1.34364E-24
XM_532632.4		0.49	1.46685E-12
JF508171.1	Timp3	0.49	8.2259E-36
XM_538410.4	Timp3	0.49	8.72417E-39
XM_543351.2	Ddx51	0.49	5.4879E-25
XM_539603.3	Gjb3	0.49	2.79148E-27
AY509607.1		0.49	4.82573E-15
XM_535897.4	Cap2	0.49	5.70997E-13
XM_003431887.1	9330182L06Rik	0.49	2.78195E-24
XM_540181.3	Slc20a1	0.49	2.72859E-39
XM_539694.3	v3	0.48	8.70245E-19
AB031276.1	Mdm2	0.48	7.18522E-40
AF100705.1		0.48	4.0037E-37
AF394784.1	Dsg3	0.48	4.59788E-38
XM_545250.4	Il1rap	0.48	2.30537E-21
XM_846545.2	Daf2	0.48	2.81735E-14
XM_546864.3	Slc7a6	0.48	1.50267E-25
XM_848524.2	Nqo1	0.48	5.51805E-18
XM_545529.3	Scrn3	0.48	7.50875E-28
XM_537579.3	Amz2	0.48	7.58602E-41
XM_003434077.1	Sec22a	0.48	3.60731E-19
XM_534069.4	Copb1	0.47	1.99832E-46
XM_850065.2		0.47	4.30154E-24
XM_003434113.1	Ece2	0.47	7.54058E-20
XM_547475.3	Blzf1	0.47	6.12899E-39
XM_849343.2	Cyld	0.47	6.26347E-25
XM_853685.2	Calu	0.46	2.9676E-28
XM_843128.2	Palld	0.46	2.34643E-20
EF450456.1	S i2	0.46	7.31581E-19
AF262963.1		0.46	2.02003E-13
XM_534520.3	Flt1	0.45	6.10277E-26
XM_531807.3	Epas1	0.45	2.03904E-18
XM_548170.4		0.45	2.95576E-18
XM_540514.2	Athl1	0.45	4.88104E-26
XM_003432574.2		0.45	2.71889E-23
XM_547922.4	Angel1	0.44	1.28819E-19
XM_539923.3	Chpf2	0.44	2.23939E-42
XM_546589.3	Plscr3	0.44	3.45025E-28
XM_543070.3	Pmepa1	0.44	1.63291E-40
XM_545229.3	Ece2	0.44	2.53398E-21

XM_539767.3	Fam160a1	0.44	1.4242E-28
AB011372.1	Slc12a4	0.44	1.12165E-51
XM_535558.3	Rbm11	0.43	1.07952E-14
XM_544148.3		0.43	4.98701E-22
Z27115.1		0.43	3.92654E-36
XM_543863.3	9630033F20Rik	0.43	7.68991E-31
XM_537805.3	Olfm1	0.42	6.5384E-32
BN000755.1		0.42	9.30244E-25
XM_540845.3	Fosl1	0.42	1.48031E-24
L28932.1		0.42	1.49827E-41
XM_003639192.1		0.41	7.39929E-61
XM_534533.3		0.40	5.08503E-32
XM_844060.1	Gata3	0.40	2.61819E-16
NM_001197022.1	Cd44	0.40	3.34044E-53
XM_845130.2		0.40	6.72984E-44
XM_003432338.1		0.40	1.15131E-38
XM_542901.3	Gpcpd1	0.40	5.25882E-33
XM_538773.4	Abca1	0.39	9.95247E-19
XM_003639193.1		0.39	1.63863E-29
XM_546674.3	Ak4	0.39	3.69679E-22
XM_543878.2	B4galnt3	0.38	5.44319E-21
XM_543730.2	Gm11821	0.38	6.295E-22
XM_544769.3	Sema7a	0.38	2.80378E-62
XM_533922.3		0.38	5.84477E-23
XM_003639882.1	Polr3d	0.38	1.32399E-60
XM_549265.3	Gpc4	0.37	1.18779E-18
XM_546059.2	F2r	0.37	6.30411E-25
XM_535151.3	9430020K01Rik	0.37	2.35996E-25
AY204568.1	Bhlhe41	0.37	7.26291E-31
XM_845643.2	Sec24a	0.37	7.32312E-64
XM_548101.2	Krt16	0.36	2.21848E-42
XM_536441.3	Ccng1	0.36	3.1425E-70
XM_846790.2	Gch1	0.35	2.15798E-37
XM_543369.3	D hc10	0.35	1.72076E-40
XM_535269.3	Map1b	0.34	7.38146E-24
XM_536261.3	Fam114a1	0.34	3.68246E-24
XM_546036.3	Edil3	0.30	3.44373E-42
XM_003639413.1	Runx2	0.29	1.44728E-27
AJ830019.1		0.28	1.2485E-101
XM_532125.3	Cdkn1a	0.28	2.7507E-107
XM_843954.2	Arf4	0.28	1.1237E-114
XM_003433852.1	Sytl1	0.26	2.55605E-73
XM_003431889.1		0.26	4.95871E-52
XM_003434922.2		0.24	1.03607E-72
XM_532641.3	Mgat4c	0.22	1.4718E-40
XM_539955.3	Plat	0.22	5.10011E-66
XM_542806.3	Esyt3	0.21	4.85391E-50
XM_846210.2	Slc10a6	0.20	4.41153E-79
DQ409210.1		0.20	2.31146E-57
AB066299.1		0.08	6.19858E-88

AF045016.1	Abcb1a	0.06	1.4838E-110

Supplementary Data-2.

Genes differentially expressed between *scrib* ^{KD} cells that are sensitive to out-competition (*scrib* ^{KD} +TET) and cells that are resistant to out-competition (*scrib* ^{RES} +TET cells).

Transcript identifier	Official gene name	FC (*scrib* ^{RES}+TET/*scrib* ^{KD}+TET)	P value
XM_534096.3	Bbox1	11.15	2.27694E-80
XM_003639011.1	Ti gl1	5.66	2.03529E-43
XM_539295.4	Tmprss11e	5.47	2.18879E-57
XM_539472.3	Gpnmb	4.60	2.5045E-224
XM_542992.2	D630003M21Rik	4.50	5.57448E-89
XM_535946.3	Lrp2	4.49	6.20822E-63
XM_536361.3	Egr2	4.18	4.93209E-40
XM_538640.3	Tgfbi	3.73	4.43951E-58
XM_847751.2	Ripply1	3.25	8.83483E-22
NM_001003282.1		3.11	2.13893E-25
XM_531763.3	Aldh1l2	2.96	7.54192E-63
XM_539002.3	Col12a1	2.93	5.11181E-56
XM_003433344.1		2.89	4.36045E-23
XM_540139.3	Capn13	2.87	3.27398E-61
XM_533253.3	Pla2g16	2.81	3.54646E-33
NM_001145151.1	Col5a2	2.81	5.23567E-25
XM_547730.3	Slc7a8	2.78	8.40589E-32
XM_003434416.1	Vcan	2.74	5.80227E-31
XM_540384.3	Cog5	2.71	1.5934E-137
XM_541746.3	Nup210	2.59	1.89515E-21
XM_538945.3	Enpp5	2.58	7.22916E-20
XM_853571.3	Tenm4	2.58	7.93708E-19
AF201729.1		2.55	1.68746E-19
XM_543883.3	Wnt5b	2.54	6.15013E-17
AB738915.1	Slc22a12	2.48	4.64399E-20
XM_536403.4	Fam213a	2.47	1.715E-112
XM_535429.3	Rasgrp1	2.46	1.05507E-50
XM_003640151.1	Btn1a1	2.43	9.21607E-26
XM_003639190.1	Cdkn1a	2.42	2.66113E-93
XM_537280.3	Fhod3	2.40	8.12173E-60
XM_534845.3	2810474O19Rik	2.37	1.82162E-94
AF149850.1		2.35	5.10426E-14
NM_001003245.1	Csf2	2.35	8.67447E-30
XM_003432501.1		2.33	1.71022E-51
XM_531781.2	Anxa2	2.28	2.31412E-22
XM_532716.3	Ddx60	2.26	3.59251E-20
XM_003433116.1	Med12l	2.25	1.06934E-25
XM_542667.3	Irs2	2.22	1.80941E-37
AF358907.1	Cldn2	2.15	1.3995E-45
DQ138952.1		2.14	4.55856E-48
AB054642.1	Ccl17	2.10	6.67813E-71
NM_001013844.1	Fosb	2.07	1.18834E-32
XM_537133.3	Pigr	2.06	5.26016E-09

AY026462.1	Il1rn	2.05	9.60212E-16
XM_538268.3	Tbc1d30	2.03	3.55096E-22
XM_850092.2		2.03	5.74899E-31
XM_538686.2	Dmrta1	2.03	9.43463E-31
XM_003434008.1	Prss12	2.01	8.30603E-17
XM_544394.3	Nlrc5	2.01	2.75257E-21
NM_001003227.1	Nr4a1	1.98	1.53558E-21
XM_548876.2	Reps2	1.97	1.17669E-13
XM_535982.4	Ttn	1.96	2.80433E-11
XM_858603.3	Foxp1	1.96	3.67159E-20
XM_546283.3	Galnt10	1.95	8.75748E-22
AY800385.2	Hipk2	1.94	1.24404E-31
XM_848680.2	Sncg	1.94	1.01035E-41
XM_547148.3	Glis2	1.94	1.82451E-16
XM_543918.2	Slc16a12	1.93	1.65984E-37
XM_534960.3	Ankrd1	1.93	4.41765E-11
XM_533551.3	Sema4d	1.93	6.38092E-23
XM_849019.2	Msln	1.92	8.58007E-13
XM_846724.1	Tspan15	1.92	2.60709E-20
AF333433.1		1.89	1.5876E-27
XM_851705.2		1.89	1.44403E-20
XM_847037.2		1.88	1.50314E-17
XM_844184.2	Upk3b	1.87	1.00782E-16
XM_003434841.1	Tmc5	1.87	4.56674E-20
XM_848544.1	Tmed6	1.87	6.67404E-10
XM_532430.3	Tspan33	1.87	9.38749E-21
XM_851664.2		1.86	1.41865E-23
XM_003433118.1	Plch1	1.86	1.75408E-15
XM_537804.3	Col5a1	1.86	1.42032E-44
XM_003432136.1	Sorbs2	1.85	5.3242E-37
XM_003431676.1	Cfb	1.85	2.36156E-12
XM_850106.2	Rap1gap	1.84	2.6392E-23
AB191461.1		1.84	6.97664E-19
EF063141.1	Ptges	1.83	8.07107E-16
XM_543290.2	Efhd1	1.83	3.75223E-12
XM_003432612.1	Trpm3	1.83	3.44753E-47
XM_537350.3	Fbln5	1.82	4.36089E-16
XM_845012.2	Orai2	1.82	1.96137E-16
AF045773.1	Adm	1.81	1.17013E-16
XM_540100.1	Slc7a15	1.81	2.75397E-33
XM_546328.2	Itga1	1.80	2.07922E-16
AB115087.1	Edn1	1.80	2.08352E-44
U96127.1	Adm	1.80	2.32742E-15
AY970669.1	Podxl	1.79	1.04835E-48
XM_543443.3	Dao	1.79	1.53153E-35
XM_534128.3	Tsc22d1	1.79	2.2619E-19
XM_534912.2	Scnn1a	1.78	2.36993E-12
XM_545847.3	Isg20	1.78	4.21829E-16
XM_860143.2	Slc6a15	1.78	6.33717E-15
XM_533037.3	Txnip	1.77	1.13785E-32

XM_844405.2	Rhobtb1	1.77	1.2476E-26
XM_003431772.1	Tmem74	1.76	1.37039E-14
XM_539783.3	Cct8	1.76	1.22743E-11
XM_531751.3	Large	1.76	3.2638E-10
XM_848450.2	Sgpp2	1.75	1.28125E-14
XM_544123.2	Trpa1	1.75	7.68561E-39
XM_532487.1	Itgb8	1.74	2.71016E-38
NM_001048086.1	Psmb9	1.74	2.67185E-17
XM_545713.3		1.74	9.7634E-21
AB194049.1		1.74	1.29392E-13
XM_545146.3	Muc20	1.74	4.03272E-24
XM_849544.2	Marcksl1	1.73	4.79311E-26
XM_003432104.1	Kat6a	1.73	5.52367E-22
XM_540076.3	Rrm2	1.73	2.18743E-41
XM_847209.2	Srf	1.73	2.54831E-21
XM_003434373.1	Slco3a1	1.72	2.67074E-20
XM_546757.3	Slc45a1	1.71	1.13191E-14
XM_543812.3	Etv6	1.71	2.58018E-23
XM_543240.3	Nkx3-1	1.71	3.50766E-12
XM_844793.2	Ivns1abp	1.71	1.43793E-41
XM_852055.2	Itgb6	1.70	1.98259E-36
XM_003434612.1	Podn	1.70	2.02005E-10
XM_544159.2	Car13	1.70	8.28677E-24
AB648939.1		1.69	5.07226E-38
XM_544391.2	Cx3cl1	1.69	3.82876E-38
XM_542979.3	Epb4.1l1	1.69	9.97386E-16
JN656393.1		1.68	9.07795E-18
XM_003639771.1	Fam107a	1.68	3.01687E-08
XM_846145.2	Egr1	1.68	6.10682E-24
XM_847458.3	Slc39a11	1.67	1.90419E-09
XM_546939.4	Cux1	1.66	9.85804E-13
XM_532878.3	Lpin1	1.66	1.38322E-23
XM_535623.3	Fras1	1.66	1.75623E-11
XM_533391.3	Zfp532	1.66	1.78295E-25
XM_543238.3	Stc1	1.65	1.31095E-31
XM_540424.3	Paip2	1.65	2.03942E-06
XM_547851.3	Syne2	1.65	6.50585E-23
XM_003434002.2	Calm1	1.65	4.28344E-15
DQ310185.1		1.65	1.55069E-29
XM_848770.2	1700011H14Rik	1.65	7.56508E-30
XM_545790.3	Muc1	1.65	4.57064E-09
XM_544011.3	Add3	1.64	9.60749E-15
XM_845400.2	Smad7	1.64	3.09987E-13
DQ517443.1		1.64	2.5022E-28
XM_546629.4	Arhgap44	1.64	1.34709E-08
XM_848227.2	C1s	1.64	2.68739E-14
AY064408.1		1.63	3.15518E-08
XM_003639406.1		1.63	8.98879E-16
XM_003432029.1		1.62	4.98891E-10
XM_532589.3	Pdzk1ip1	1.62	7.93935E-20

AF345933.1		1.62	3.44237E-13
AF178116.1		1.62	3.98043E-22
XM_538324.3	Celsr1	1.62	5.07684E-15
XM_003434496.1		1.62	4.01227E-11
XM_545014.3	Npnt	1.62	2.14139E-16
XM_532713.3	Klhl2	1.62	4.62812E-17
AF211257.1	Fgfr2	1.61	1.25782E-28
XM_547695.3	Zbtb7c	1.61	2.37027E-09
XM_843373.2	Agap1	1.61	6.03325E-22
XM_536212.3	Ctsh	1.61	8.42682E-33
XM_003432071.1	3110062M04Rik	1.61	2.78268E-11
XM_847453.3	Arid1a	1.61	1.85462E-09
XM_548419.3	Prrx2	1.61	2.85047E-08
NM_001197189.1	Slc35c1	1.61	3.32155E-15
XM_532560.4	Oscp1	1.61	2.96159E-08
XM_003434303.1	Zbed6	1.61	4.74571E-17
JN656391.1	Tap1	1.61	2.01743E-20
XM_003639542.1	Mll3	1.61	9.89072E-26
XM_535960.3	Cybrd1	1.60	3.56011E-10
AF023617.1	Tjp3	1.60	3.02525E-17
XM_545778.3	Olfml2b	1.60	7.47239E-28
XM_539370.3	Plx 4	1.60	1.26097E-08
XM_854718.2	3110062M04Rik	1.60	1.5501E-11
XM_003434597.1	Frmd4b	1.59	1.01259E-09
XM_545721.4	Disp1	1.59	1.84409E-15
XM_003432185.1		1.59	5.67952E-22
XM_544383.3	Mmp15	1.59	1.4796E-15
XM_847899.2	Glce	1.59	2.75763E-11
XM_844022.2	Rassf4	1.59	3.49698E-12
XM_844782.2	Anxa1	1.59	7.74421E-31
XM_003432184.1		1.59	4.66444E-35
XM_534170.3		1.58	6.10564E-23
XM_849866.2	Nfatc4	1.58	7.77542E-11
XM_532171.3	Tram2	1.58	6.5918E-08
XM_549250.1	Elf4	1.58	8.80403E-08
XM_003435109.1	Dctn6	1.58	7.78595E-11
XM_539158.3		1.57	1.75367E-14
XM_849853.2	Sobp	1.57	3.11273E-12
XM_546181.3	Zmiz1	1.57	2.71362E-21
XM_003434473.1	Slit3	1.57	5.57254E-20
XM_847992.2	Ncor2	1.57	8.39761E-24
XM_003435368.1		1.57	1.9623E-18
XM_532117.3	Satb1	1.57	1.20126E-06
XM_538256.3	Ctdsp2	1.57	7.81299E-16
XM_003638944.1		1.57	6.44773E-07
XM_003434864.1	Crebbp	1.57	3.07951E-25
XM_851394.1	Arhgap31	1.56	1.47135E-29
XM_546495.3	Bcl9l	1.56	5.52197E-18
XM_535240.4		1.56	4.14926E-18
XM_536182.3		1.56	4.32201E-22

XM_849458.2	Krt7	1.56	1.28242E-10
XM_847879.2	Tmem37	1.56	2.51472E-10
XM_540506.3	Sema3e	1.55	1.65244E-25
XM_003638813.1	Atf5	1.55	8.26276E-11
XM_533525.3	Aldh1a1	1.55	5.00829E-30
XM_003639229.1	Cdh24	1.55	7.22081E-11
XM_003432240.1		1.55	3.34794E-11
XM_003433173.1		1.55	1.50558E-08
XM_847020.2	Rhob	1.54	9.96267E-15
XM_534901.3	C1ra	1.54	1.63975E-11
NM_001197095.1	Serpine1	1.54	2.73942E-15
XM_542318.2	Relt	1.54	7.45886E-09
XM_843488.2	Foxp4	1.54	9.15544E-10
XM_544332.3	Dpysl3	1.54	2.55283E-23
XM_003639829.1	Ppp2r3a	1.54	5.21213E-24
XM_003432561.1		1.53	3.58479E-18
NM_001013416.1	Brca1	1.53	6.08108E-14
XM_543406.3	Slc24a6	1.53	2.47813E-07
XM_533516.3	Tle1	1.53	2.397E-15
XM_544900.1	Zbtb21	1.53	2.96991E-08
XM_003639845.1	Ptgis	1.53	3.02635E-09
XM_856020.2	Map2	1.53	5.79982E-13
JN656398.1	Lrrc1	1.52	1.30943E-05
XM_541154.3		1.52	3.51128E-20
XM_859340.2		1.52	2.10212E-12
XM_543225.3	Scara3	1.52	8.48479E-18
NM_001172543.1	Peg10	1.52	5.6107E-12
XM_003435518.1	Calu	1.52	8.07591E-06
XM_545639.3	Tns1	1.52	9.49077E-25
XM_846706.2	Gse1	1.51	3.89642E-12
XM_847525.2		1.51	5.41258E-08
XM_003433936.1		1.51	8.87691E-07
XM_545692.2	Slc45a3	1.51	9.36915E-06
XM_540856.3	Fam89b	1.51	6.90733E-10
XM_537487.3	Plekhh1	1.51	9.67149E-06
NM_001195154.1		1.51	4.45863E-13
AY292464.1	LOC101056437	1.51	2.44814E-12
XM_536095.3	Plekha6	1.51	1.31119E-11
XM_537333.2	Apcdd1	1.51	1.25672E-12
XM_548413.3	Abl1	1.51	1.47075E-16
XM_532891.3	Ncoa1	1.51	2.91187E-06
XM_849496.2	Tmem54	1.51	1.59463E-06
XM_857127.2		1.50	4.4691E-14
NM_001003295.1	Hspb1	1.50	4.01247E-19
AJ388536.1		1.50	1.13995E-12
XM_535507.3	Car12	1.50	1.34911E-18
XM_540177.3	Fbln7	1.50	4.49716E-14
XM_542963.3	E2f1	1.50	1.56278E-09
XM_540101.3	Dennd1a	1.50	9.95653E-15
XM_844754.2	Plekho1	1.50	2.82549E-16

XM_003639180.1	Cenpf	1.50	4.50526E-19
XM_853033.2	Rcan1	1.50	1.61712E-06
XM_845170.2	Zc3h12a	1.49	1.93913E-08
XM_535735.3	Pvrl3	1.49	5.14186E-11
XM_003432616.1	Kank1	1.49	5.55981E-15
XM_003435522.1	Hs6st2	1.49	1.5158E-10
XM_845303.2	Insig1	1.49	3.21597E-20
XM_003432467.1		1.49	8.34949E-12
XM_003432847.2	Mgat4b	1.49	5.68671E-06
XM_543166.3	Spata13	1.49	9.21252E-08
XM_845406.2	Fstl1	1.49	7.49285E-20
XM_547735.3	Zfhx2	1.49	7.16748E-07
XM_536734.3	Rere	1.49	1.13107E-21
XM_534183.3	Col4a2	1.49	2.63015E-24
EU036633.1	Tnfsf10	1.49	9.99856E-12
XM_547747.3	Nynrin	1.49	1.78822E-08
XM_003433866.1	Arhgef10l	1.49	5.31915E-08
AB636469.1	Vldlr	1.49	2.53403E-17
XM_541589.3	Cic	1.48	1.92771E-17
XM_542984.3	9830001H06Rik	1.48	6.16788E-06
XM_543682.3	Kcnh3	1.48	1.90961E-05
XM_547007.3	Iqce	1.48	7.16352E-09
XM_003432323.1	Fam89b	1.48	1.50036E-09
XM_852283.2	Acsl1	1.48	6.58059E-23
XM_003433593.2	Mki67	1.48	2.98424E-14
XM_845203.2	Hivep2	1.48	4.63923E-11
XM_536720.4	Prdm16	1.47	2.68758E-05
XM_546156.3	Ddit4	1.47	3.898E-11
XM_538677.3	Mllt3	1.47	1.4333E-09
AF167075.2	Dsg3	1.47	1.09842E-20
XM_546721.3	Ttll10	1.47	2.2171E-09
XM_843925.2	Dap	1.47	2.01548E-15
XM_003639885.1	Bmp1	1.47	1.03758E-14
XM_536144.3	Rpl35	1.47	9.79124E-08
XM_847901.2	Morf4l2	1.47	4.97367E-07
NM_001003229.1	Bgn	1.47	4.19115E-12
XM_533114.3		1.47	1.32693E-10
XM_533089.4	Lamb1	1.46	7.05227E-24
XM_541516.3	Sphk2	1.46	9.91701E-07
M95495.1	Slc6a6	1.46	6.98371E-17
XM_003432611.1		1.46	7.65773E-07
XM_845240.2	Sfmbt2	1.46	1.5656E-11
AY156692.1	Ctss	1.46	2.41576E-10
XM_846619.2	Ssh1	1.46	2.91964E-19
XM_546145.3	Eif4ebp2	1.46	9.82258E-20
XM_537553.3	Evl	1.46	2.3362E-09
XM_003435094.1	Trappc4	1.46	8.35761E-10
XM_548064.4	Hdac5	1.46	1.5516E-10
XM_844202.2	Rcan1	1.45	4.94237E-18
XM_844186.1	Gas1	1.45	5.38447E-06

XM_847382.2	Atf3	1.45	2.88459E-17
XM_003639315.1		1.45	4.91255E-09
XM_543946.3	Lcor	1.45	4.02885E-08
XM_846419.2	Zfp362	1.45	1.07873E-14
XM_543403.3	Rasal1	1.45	8.96437E-13
XM_848051.2	Gabarapl1	1.45	1.11221E-17
XM_541795.3	Bhlhe40	1.45	7.13405E-16
FR775794.1		1.45	4.89305E-07
XM_543298.3	Sh3bp4	1.45	4.84469E-20
XM_847234.1		1.44	3.16113E-08
XM_003639657.1	Ah k	1.44	1.60182E-15
XM_003432485.1	R3hdm1	1.44	1.76616E-19
XM_003431469.1		1.44	1.18525E-08
XM_003433095.1	Col4a1	1.44	2.94395E-19
XM_003639120.1	Gdpd3	1.44	4.21497E-06
XM_538511.3	Slc1a4	1.44	1.12417E-09
XM_546237.4	Sh3pxd2b	1.44	1.21129E-10
X83591.1	Pax8	1.44	3.94964E-07
AY509607.1		1.44	2.98703E-07
XM_533102.3	Mll5	1.44	2.55172E-18
AY069922.1	Sult1a1	1.44	2.94531E-16
XM_003434092.2	BC016579	1.44	2.83009E-07
XM_544912.4	Sik1	1.44	2.92076E-12
XM_542172.4	Pip5k1c	1.44	6.29994E-06
XM_850436.3	Crip2	1.44	8.30888E-15
XM_538721.3	Hint2	1.44	2.21903E-14
XM_003433519.1	Dusp11	1.44	2.663E-05
XM_848109.2	Ssbp3	1.44	1.21893E-09
XM_542889.2		1.44	2.42425E-10
XM_850020.2	Il22ra1	1.44	9.19985E-07
XM_843509.2	Ttc17	1.43	9.92585E-15
AF133248.1	Vegfa	1.43	9.31554E-15
XM_003640219.1	Med12	1.43	2.69134E-10
XM_845618.2	Vopp1	1.43	4.6519E-06
XM_844386.2	Gatsl2	1.43	0.000151841
XM_544699.4	Cgnl1	1.43	5.71173E-09
XM_539852.3	Zyx	1.43	4.37184E-18
XM_843712.2	Rab17	1.43	0.00016624
XM_541153.2	Zbtb2	1.43	8.83017E-08
XM_847302.2	Mxi1	1.43	5.10278E-13
XM_541510.2	Fgf21	1.43	2.9389E-12
XM_003639801.1	Tsc22d1	1.43	1.42049E-19
XM_844217.2	Csad	1.43	4.93201E-10
XM_003639342.1	Ttll1	1.43	2.58675E-07
XM_546807.3	Phf21a	1.43	3.40789E-05
XM_003433284.1		1.43	2.16907E-16
XM_543243.3	LOC100862375	1.43	1.77321E-16
XM_540825.3	Pcx	1.43	6.26076E-12
XM_003638941.1		1.43	9.36138E-16
AB043895.5	Brca2	1.43	3.09577E-12

XM_003433624.1	Ndufs6	1.43	3.21121E-05
XM_544006.3	Slk	1.43	5.02362E-06
XM_548371.3	Hspa5	1.43	7.82417E-10
XM_003433115.2		1.42	7.4178E-08
XM_844500.2	Arid5b	1.42	1.59622E-12
JQ733515.1		1.42	9.06652E-17
XM_541709.2	Kctd15	1.42	1.09308E-07
XM_003432618.1		1.42	2.5578E-17
XM_535560.2	Nrip1	1.42	1.02867E-06
XM_532604.3	Plk3	1.42	1.53911E-11
XM_003431496.1	Ep300	1.42	6.00756E-19
XM_003435150.1	Tnrc6c	1.42	2.82377E-08
XM_843994.2	Srebf2	1.42	9.96584E-15
XM_534424.3	Mybl2	1.42	3.69014E-17
D29807.1	Unk	1.42	1.90577E-06
XM_543244.3	Loxl2	1.42	5.7454E-17
XM_533210.3	Chka	1.42	2.26575E-07
XM_843875.2		1.42	2.76833E-09
XM_003431746.1		1.42	2.33581E-09
XM_003638947.1	Spen	1.41	7.50098E-17
XM_544368.3	Marveld2	1.41	4.36134E-07
XM_003640047.1	B2m	1.41	1.6436E-15
XM_003639960.1	Tuba1c	0.71	5.84885E-19
XM_544107.1	Nipbl	0.71	8.74504E-10
JX306093.1	Adamts10	0.71	2.15092E-05
XM_533270.3	Fads3	0.70	3.25109E-16
XM_536876.3	Baiap2l1	0.70	1.10129E-15
XM_850442.2	Anxa10	0.70	3.97979E-08
XM_848025.2	Bag2	0.70	1.14331E-07
XM_846481.2	Itpa	0.70	5.00448E-16
AY422991.1	Npas2	0.70	2.54694E-22
XM_003432524.1	Sgk1	0.70	5.03875E-18
XM_543458.3	Tpst2	0.70	3.33735E-05
XM_545250.4	Il1rap	0.70	9.25508E-08
XM_003435004.1		0.70	1.76177E-10
XM_544179.3	Cdh17	0.70	1.01619E-14
XM_003432793.1		0.70	1.00931E-05
NM_001197096.1	Ppp3ca	0.70	2.22928E-11
XM_003434714.1	Fhod1	0.70	5.14063E-06
XM_536702.3	Magoh	0.70	5.19572E-16
XM_845324.3	Lix1l	0.69	1.72497E-08
XM_003432574.2		0.69	1.20899E-07
XM_533912.3	Tmem205	0.69	7.2803E-14
XM_539605.2	Gjb5	0.69	1.06777E-09
XM_003432315.1		0.69	9.50818E-06
XM_536010.3	Tmeff2	0.69	5.76378E-11
AF016649.2	Map3k1	0.69	4.08836E-18
XM_537128.3	Ddx59	0.69	1.41052E-19
XM_533922.3		0.69	1.92514E-18
XM_533301.3	Bbs7	0.69	9.11928E-09

L28932.1		0.69	3.44636E-13
XM_535987.3		0.69	1.26362E-08
XM_535637.3	Agpat9	0.68	1.54761E-09
NM_001197022.1	Cd44	0.68	4.85428E-17
XM_545701.3	Rgs2	0.68	6.81569E-11
XM_538032.3	Praf2	0.68	6.2862E-21
XM_846002.2	Pla2g7	0.68	7.76692E-22
XM_003639615.1	Ppard	0.68	8.48541E-10
XM_850121.2	Tube1	0.68	2.92651E-11
XM_003432942.1	Lpcat3	0.68	9.54901E-10
XM_843368.2	Tubb4b	0.68	1.57827E-22
AF155148.1	Flt3l	0.68	1.61432E-07
XM_539278.3	Aasdh	0.68	2.41126E-06
NM_001197170.1	Col9a2	0.68	6.33977E-12
JQ250035.1		0.68	2.1854E-10
XM_846683.2	1190005I06Rik	0.68	4.43127E-15
XM_545529.3	Scrn3	0.68	2.36408E-11
XM_548170.4		0.67	1.50505E-06
XM_535006.3	Col17a1	0.67	3.27932E-24
XM_536077.3	Tuba4a	0.67	8.08272E-18
XM_844206.2		0.67	4.54223E-08
XM_535998.3	Bmpr1b	0.67	5.70169E-25
NM_001253720.1		0.67	3.25231E-23
XM_848175.1	Tubb6	0.67	5.64127E-12
XM_541772.2	Irak2	0.67	1.47344E-07
XM_003434922.2		0.67	1.18887E-11
XM_542216.4	Palm	0.67	6.05998E-06
AY455801.1		0.67	5.48644E-15
XM_847318.2	Elmo1	0.67	4.31702E-20
AF394784.1	Slc1a1	0.66	1.1032E-20
XM_546696.2	Slc20a1	0.66	6.8448E-06
XM_847416.2	Syngr1	0.66	4.02813E-09
XM_003432338.1		0.66	3.04634E-09
XM_535651.2	Ppm1k	0.66	1.3211E-13
NM_001003112.1	Uaca	0.66	1.2913E-18
XM_846177.2	9930012K11Rik	0.66	3.47769E-11
XM_538748.3	Tbc1d2	0.66	8.84025E-18
XM_847117.2	Id1	0.66	4.88747E-16
AY057077.1	Tpmt	0.66	2.05653E-09
XM_536768.3	Gcsh	0.66	3.49905E-13
XM_534148.3	Lmo7	0.66	1.61924E-20
XM_548445.3	St6gal c6	0.66	7.6871E-17
XM_533524.3	Rdh10	0.65	9.18439E-31
XM_850178.2	R set2a	0.65	3.76072E-18
XM_003434708.1		0.65	1.46178E-07
XM_003433614.1		0.65	6.70133E-17
XM_531678.3	Tspan8	0.64	1.73687E-15
XM_848524.2	Nqo1	0.64	1.49213E-09
NM_001253721.1	Phyh	0.64	8.96952E-12
XM_540914.3	Hyal1	0.64	6.33185E-09

XM_536530.3	Aplp2	0.64	6.75703E-25
XM_532542.3	Exo5	0.64	1.97937E-19
XM_850064.2	S100a14	0.64	1.00056E-25
XM_003435145.1	Pir	0.64	2.02874E-07
XM_845599.2	Tfap2a	0.64	2.77784E-09
XM_845057.2	Ralgps2	0.64	2.67403E-15
XM_849226.3	Rgs11	0.63	2.35753E-12
NM_001003198.1	Tuba1b	0.63	7.73056E-22
XM_537579.3	Amz2	0.63	3.35506E-27
XM_544713.3	Lactb	0.63	2.23153E-12
XM_539257.4	Nipal1	0.62	2.2421E-12
DQ975208.1	Ceacam1	0.62	6.71428E-21
XM_003434948.1	Sh3bp5	0.62	5.58624E-14
XM_532262.3	Ddo	0.62	2.45633E-07
XM_539603.3	Gjb3	0.61	5.05127E-18
XM_853765.2	Myof	0.61	9.11725E-29
XM_544163.3	Cpne3	0.61	4.38992E-16
XM_003431466.1	Cpm	0.61	4.0514E-10
XM_003434024.1	Spp1	0.61	2.0572E-21
XM_541571.4	Bcam	0.60	2.39135E-36
XM_538599.2	Lox	0.60	2.36935E-37
XM_540845.3	Fosl1	0.60	2.40818E-13
AB011372.1	Slc12a4	0.59	2.53816E-39
XM_546036.3	Edil3	0.59	6.09669E-11
XM_545846.3	Aen	0.59	1.38268E-29
XM_846790.2	Gch1	0.59	4.49272E-14
XM_536545.3	Sorl1	0.59	3.29276E-22
XM_534237.3	Tgfbr2	0.58	3.92799E-40
XM_003639937.1	Eno2	0.58	5.59344E-21
XM_537587.3	1810010H24Rik	0.58	4.18657E-09
DQ195101.1		0.58	1.4037E-41
XM_548004.3	Jag2	0.58	2.53853E-26
XM_003434023.1		0.58	1.49451E-36
XM_539915.3	Atg9b	0.57	7.28771E-23
XM_846210.2	Cd74	0.57	1.93039E-14
DQ409210.1		0.57	1.19607E-10
XM_003431889.1		0.57	8.81639E-15
XM_003639810.1	Tex30	0.57	2.99856E-20
XM_533382.3	Serpinb5	0.57	7.01457E-23
XM_537170.3	Pla2g4a	0.56	3.05086E-07
XM_536441.3	Ccng1	0.55	6.07385E-42
AB031276.1	Mdm2	0.55	4.60964E-45
XM_547976.3	Degs2	0.55	2.95113E-17
XM_003434976.1		0.54	1.45113E-18
XM_533427.3	Gm6314	0.54	5.03106E-14
XM_535330.3	Gm2a	0.54	1.85607E-43
XM_537061.4	Dpyd	0.53	1.34717E-49
XM_848021.2	Ppif	0.53	3.95722E-45
XM_003435547.1	Zfp185	0.53	1.68335E-11
AY646195.1		0.53	2.34097E-14

XM_537805.3	Olfm1	0.53	5.82233E-22
AF143503.1		0.52	4.41899E-15
AY044905.1	Ptgs2	0.52	1.2848E-08
AF100705.1		0.51	7.189E-53
XM_003433595.1	Sorbs1	0.51	5.88078E-11
XM_549046.3	Heph	0.51	2.81051E-28
XM_543048.1		0.51	1.899E-15
XM_546184.3	Sftpd	0.51	2.17403E-46
XM_535151.3	9430020K01Rik	0.50	7.68292E-15
AF314533.1	Il13ra2	0.50	4.08557E-14
XM_846545.2	Jph1	0.50	8.13762E-14
XM_546505.3	Tmprss4	0.49	1.72647E-14
XM_543863.3	9630033F20Rik	0.49	1.29532E-28
XM_845130.2		0.48	1.35011E-41
XM_843558.2	Pter	0.46	1.14862E-20
XM_544130.3	Epha2	0.46	8.21544E-14
XM_859070.2	Serpine2	0.45	7.17629E-29
AJ315401.1		0.45	3.2868E-68
AJ830019.1		0.42	1.91548E-85
XM_532125.3		0.42	1.64047E-93
XM_532641.3	Mgat4c	0.40	5.51282E-19
XM_543369.3	D hc10	0.39	9.60905E-41
XM_542806.3	Esyt3	0.39	1.63388E-25
XM_548101.2	Krt16	0.39	2.42365E-48
XM_541532.4		0.35	2.42249E-51
XM_843487.2	Irx2	0.32	2.84685E-25
XM_003433320.1	Pid1	0.31	6.34337E-34
XM_003433852.1	Sytl1	0.28	4.67148E-92
AB066299.1		0.20	3.55504E-55
AF045016.1	Abcb1a	0.17	4.12105E-72

Supplementary Data-3.

List of genes that are differentially expressed between *scrib* ^{KD} +TET cells with respect to both control (*scrib* ^{KD} -TET) and *scrib* ^{RES} +TET cells.

Transcript identifier	Official gene name	FC (*scrib* ^{KD}-TET /*scrib* ^{KD}+TET)	P value	FC (*scrib* ^{RES}+TET /*scrib* ^{KD}+TET)	P value
XM_847751.2	Ripply1	13.09	9E-156	3.25	9E-22
XM_003434416.1	Vcan	8.72	4E-197	2.74	6E-31
AB738915.1	Slc22a12	7.00	3E-111	2.48	5E-20
NM_001003282.1		6.43	1E-119	3.11	2E-25
XM_535946.3	Lrp2	6.32	1E-106	4.49	6E-63
XM_542992.2	D630003M21Rik	5.41	1E-84	4.50	6E-89
XM_532430.3	Tspan33	5.08	4E-125	1.87	9E-21
XM_003434612.1	Podn	4.88	7E-93	1.70	2E-10
DQ138952.1		4.26	9E-120	2.14	5E-48
XM_547730.3	Slc7a8	4.26	8E-48	2.78	8E-32
XM_539370.3	Plx 4	4.07	3E-70	1.60	1E-08
AF358907.1	Cldn2	3.96	1E-100	2.15	1E-45
XM_548371.3	Egfl7	3.70	5E-109	1.43	8E-10
NM_001003227.1	Nr4a1	3.58	1E-39	1.98	2E-21
AB191461.1		3.48	1E-69	1.84	7E-19
AF045773.1	Adm	3.46	6E-65	1.81	1E-16
U96127.1	Adm	3.46	5E-62	1.80	2E-15
XM_537133.3	Pigr	3.40	7E-31	2.06	5E-09
XM_533253.3	Pla2g16	3.33	3E-39	2.81	4E-33
XM_844782.2	Rdh10	3.15	4E-98	1.59	8E-31
XM_003432847.2	Palm3	3.07	4E-39	1.49	6E-06
XM_531781.2	Npas2	3.02	3E-28	2.28	2E-22
NM_001195154.1		2.96	4E-70	1.51	4E-13
XM_003434496.1		2.93	3E-48	1.62	4E-11
XM_536010.3	Tmeff2	2.92	2E-72	0.69	6E-11
AF023617.1	Tjp3	2.89	4E-67	1.60	3E-17
XM_003639180.1	Cenpf	2.87	1E-83	1.50	5E-19
XM_849019.2	Msln	2.87	9E-43	1.92	9E-13
XM_003639229.1	Cdh24	2.84	4E-49	1.55	7E-11
AF178116.1		2.69	4E-64	1.62	4E-22
XM_533037.3	Txnip	2.64	8E-59	1.77	1E-32
XM_543238.3	Stc1	2.59	3E-69	1.65	1E-31
D29807.1	Sult1a1	2.56	6E-39	1.42	2E-06
XM_003432240.1		2.55	2E-35	1.55	3E-11
XM_003640151.1	Btn1a1	2.50	4E-22	2.43	9E-26
XM_003433118.1	Plch1	2.49	6E-25	1.86	2E-15
AY064408.1		2.48	1E-27	1.63	3E-08
XM_847879.2	Tmem37	2.47	1E-37	1.56	3E-10
XM_533525.3	Aldh1a1	2.47	3E-65	1.55	5E-30
AY069922.1	Sult1a1	2.44	4E-59	1.44	3E-16
XM_532560.4	Oscp1	2.43	6E-26	1.61	3E-08
XM_540076.3	Rrm2	2.42	4E-66	1.73	2E-41
XM_541746.3	Nup210	2.40	4E-16	2.59	2E-21

XM_847234.1		2.37	2E-30	1.44	3E-08
XM_849866.2	Anks6	2.36	2E-31	1.58	8E-11
XM_538268.3	Tbc1d30	2.35	1E-27	2.03	4E-22
NM_001197189.1	Slc35c1	2.35	1E-39	1.61	3E-15
XM_844405.2	Rhobtb1	2.35	4E-46	1.77	1E-26
XM_532589.3	Pdzk1ip1	2.34	2E-40	1.62	8E-20
XM_532891.3	Ncoa1	2.34	6E-27	1.51	3E-06
XM_543918.2	Slc16a12	2.34	1E-45	1.93	2E-37
XM_848544.1	Tmed6	2.33	3E-21	1.87	7E-10
XM_003432071.1	3110062M04Rik	2.33	2E-30	1.61	3E-11
XM_847037.2		2.31	8E-27	1.88	2E-17
XM_858603.3	Foxp1	2.30	1E-27	1.96	4E-20
XM_003435109.1	Nfatc4	2.27	9E-28	1.58	8E-11
XM_533210.3	Chka	2.25	3E-29	1.42	2E-07
XM_854718.2	3110062M04Rik	2.24	7E-29	1.60	2E-11
XM_848770.2	1700011H14Rik	2.24	3E-49	1.65	8E-30
XM_542979.3	Epb4.1l1	2.23	3E-28	1.69	1E-15
XM_540139.3	Capn13	2.23	2E-27	2.87	3E-61
EF063141.1	Ptges	2.23	1E-24	1.83	8E-16
NM_001013844.1	Fosb	2.22	5E-22	2.07	1E-32
XM_543443.3	Dao	2.22	3E-42	1.79	2E-35
XM_538677.3	Mllt3	2.22	9E-31	1.47	1E-09
XM_853571.3	Tenm4	2.21	2E-09	2.58	8E-19
XM_003431466.1	Cpm	2.18	3E-27	0.61	4E-10
XM_849853.2	Sobp	2.15	3E-18	1.57	3E-12
XM_534912.2	Scnn1a	2.13	2E-17	1.78	2E-12
XM_532487.1	Itgb8	2.13	7E-44	1.74	3E-38
XM_543403.3	Rasal1	2.12	4E-37	1.45	9E-13
XM_003433593.2	Mki67	2.12	3E-30	1.48	3E-14
XM_544383.3	Mmp15	2.11	1E-23	1.59	1E-15
XM_003640219.1	Med12	2.10	5E-30	1.43	3E-10
XM_543682.3	Kcnh3	2.09	1E-17	1.48	2E-05
AY156692.1	Ctss	2.09	1E-28	1.46	2E-10
FR775794.1		2.09	9E-21	1.45	5E-07
AF345933.1		2.08	3E-24	1.62	3E-13
AF211257.1	Fgfr2	2.08	7E-41	1.61	1E-28
XM_003638813.1	Atf5	2.07	1E-25	1.55	8E-11
XM_544123.2	Trpa1	2.07	1E-40	1.75	8E-39
XM_547148.3	Glis2	2.06	2E-15	1.94	2E-16
XM_543166.3	Spata13	2.06	2E-18	1.49	9E-08
XM_545778.3	Olfml2b	2.04	6E-38	1.60	7E-28
XM_535560.2	Nrip1	2.03	5E-23	1.42	1E-06
XM_846419.2	Zfp362	2.02	4E-27	1.45	1E-14
XM_003639845.1	Ptgis	2.02	6E-21	1.53	3E-09
NM_001172543.1	Peg10	2.02	4E-27	1.52	6E-12
XM_546495.3	Bcl9l	2.01	6E-18	1.56	6E-18
XM_537333.2	Apcdd1	2.00	1E-25	1.51	1E-12
XM_846724.1	Tspan15	1.99	3E-19	1.92	3E-20
XM_542216.4	Palm	1.99	1E-19	0.67	6E-06
XM_850106.2	Rap1gap	1.96	2E-23	1.84	3E-23

XM_003432104.1	Kat6a	1.96	8E-25	1.73	6E-22
XM_849496.2	Tmem54	1.96	4E-17	1.51	2E-06
XM_852283.2	Acsl1	1.95	6E-39	1.48	7E-23
XM_003432501.1		1.95	3E-25	2.33	2E-51
XM_544159.2	Car13	1.93	2E-28	1.70	8E-24
XM_003434597.1	Fam211a	1.92	3E-19	1.59	1E-09
XM_847302.2	Mxi1	1.91	1E-27	1.43	5E-13
XM_546757.3	Slc45a1	1.91	2E-17	1.71	1E-14
XM_544912.4	Sik1	1.90	6E-16	1.44	3E-12
XM_542889.2		1.89	2E-24	1.44	2E-10
XM_849544.2	Marcksl1	1.88	4E-24	1.73	5E-26
XM_542172.4	Pip5k1c	1.88	2E-15	1.44	6E-06
XM_848450.2	Sgpp2	1.87	2E-14	1.75	1E-14
XM_547695.3	Zbtb7c	1.87	2E-14	1.61	2E-09
XM_845170.2	Zc3h12a	1.86	2E-13	1.49	2E-08
XM_547007.3	Iqce	1.83	1E-16	1.48	7E-09
XM_543240.3	Nkx3-1	1.83	4E-14	1.71	4E-12
AF167075.2	Slc1a1	1.83	2E-30	1.47	1E-20
XM_534845.3	2810474O19Rik	1.83	3E-30	2.37	2E-94
XM_003639190.1		1.82	8E-13	2.42	3E-93
XM_532713.3	Klhl2	1.81	4E-21	1.62	5E-17
XM_847901.2	Hnf1b	1.81	3E-12	1.47	5E-07
XM_534128.3	Tsc22d1	1.81	2E-13	1.79	2E-19
XM_534901.3	C1ra	1.80	1E-18	1.54	2E-11
XM_844184.2	Upk3b	1.80	2E-12	1.87	1E-16
XM_003639406.1		1.78	6E-18	1.63	9E-16
XM_543406.3	Slc24a6	1.78	3E-11	1.53	2E-07
XM_533114.3	Cdc42ep4	1.78	4E-19	1.47	1E-10
XM_545146.3	Muc20	1.77	8E-19	1.74	4E-24
XM_003639801.1	Tsc22d1	1.76	4E-24	1.43	1E-19
XM_859340.2		1.72	6E-11	1.52	2E-12
JN656398.1		1.72	7E-08	1.52	1E-05
XM_536095.3	Plekha6	1.72	8E-13	1.51	1E-11
XM_533391.3	Zfp532	1.72	9E-20	1.66	2E-25
XM_543225.3	Scara3	1.71	6E-21	1.52	8E-18
XM_548064.4	Hdac5	1.71	7E-16	1.46	2E-10
NM_001013416.1	Brca1	1.71	2E-17	1.53	6E-14
XM_003435518.1	Clcn5	1.71	2E-09	1.52	8E-06
XM_003433866.1	Arhgef10l	1.71	8E-12	1.49	5E-08
XM_003434864.1	Crebbp	1.70	5E-14	1.57	3E-25
X83591.1	Pax8	1.70	9E-12	1.44	4E-07
XM_848051.2	Gabarapl1	1.69	3E-22	1.45	1E-17
JN656393.1		1.68	2E-13	1.68	9E-18
XM_847209.2	Srf	1.68	5E-16	1.73	3E-21
JN656391.1	Tap1	1.67	3E-18	1.61	2E-20
XM_547735.3	Zfhx2	1.66	4E-07	1.49	7E-07
XM_540856.3	Fam89b	1.66	3E-12	1.51	7E-10
XM_003432323.1	Fam89b	1.66	3E-13	1.48	2E-09
XM_003433284.1		1.64	2E-19	1.43	2E-16
XM_003639315.1	Dab2ip	1.64	3E-11	1.45	5E-09

XM_003432616.1	Kank1	1.64	2E-16	1.49	6E-15
XM_003432467.1		1.64	3E-13	1.49	8E-12
XM_546807.3	Osgin1	1.63	4E-08	1.43	3E-05
XM_534424.3	Mybl2	1.63	2E-20	1.42	4E-17
AB194049.1		1.62	1E-07	1.74	1E-13
XM_541795.3	Bhlhe40	1.61	7E-15	1.45	7E-16
XM_003432029.1		1.61	2E-08	1.62	5E-10
XM_545721.4	Disp1	1.61	1E-12	1.59	2E-15
XM_544394.3	Nlrc5	1.60	4E-09	2.01	3E-21
XM_845012.2	Orai2	1.60	2E-09	1.82	2E-16
XM_533516.3	Tle1	1.59	3E-13	1.53	2E-15
XM_538324.3	Celsr1	1.59	1E-08	1.62	5E-15
NM_001048086.1	Psmb9	1.59	8E-11	1.74	3E-17
XM_003639120.1	Gdpd3	1.58	1E-08	1.44	4E-06
DQ195101.1		1.58	3E-13	0.58	1E-41
XM_003434023.1		1.58	5E-13	0.58	1E-36
XM_549250.1	Elf4	1.57	2E-06	1.58	9E-08
XM_546939.4	Cux1	1.57	1E-08	1.66	1E-12
XM_536182.3		1.57	5E-14	1.56	4E-22
XM_540424.3	Hid1	1.57	2E-05	1.65	2E-06
AF333433.1		1.56	1E-10	1.89	2E-27
XM_548413.3	Abl1	1.56	8E-13	1.51	1E-16
XM_536734.3	Rere	1.56	3E-10	1.49	1E-21
XM_541589.3	Cic	1.55	7E-09	1.48	2E-17
XM_003434841.1	Tmc5	1.55	6E-09	1.87	5E-20
XM_546721.3	Ttll10	1.54	6E-10	1.47	2E-09
XM_543812.3	Etv6	1.54	1E-10	1.71	3E-23
AY970669.1	Podxl	1.54	9E-13	1.79	1E-48
XM_532604.3	Plk3	1.54	1E-10	1.42	2E-11
XM_545847.3	Isg20	1.53	4E-08	1.78	4E-16
XM_003639657.1	Ah k	1.53	5E-13	1.44	2E-15
NM_001003245.1	Csf2	1.52	1E-06	2.35	9E-30
XM_538686.2	Dmrta1	1.52	6E-10	2.03	9E-31
NM_001003295.1	Hspb1	1.52	8E-12	1.50	4E-19
XM_538256.3	Ctdsp2	1.52	2E-10	1.57	8E-16
XM_003434473.1	Slit3	1.50	3E-08	1.57	6E-20
XM_535735.3	Pvrl3	1.50	2E-09	1.49	5E-11
XM_847458.3	Slc39a11	1.49	6E-06	1.67	2E-09
XM_843488.2	v1	1.49	4E-05	1.54	9E-10
XM_003435094.1	Homez	1.48	1E-07	1.46	8E-10
XM_003639829.1	Ppp2r3a	1.47	3E-12	1.54	5E-24
XM_533102.3	Mll5	1.46	1E-09	1.44	3E-18
XM_850442.2	Anxa10	1.46	1E-08	0.70	4E-08
M95495.1	Slc6a6	1.45	4E-10	1.46	7E-17
XM_857127.2		1.45	2E-07	1.50	4E-14
XM_547851.3	Syne2	1.45	3E-09	1.65	7E-23
XM_003433936.1		1.44	6E-05	1.51	9E-07
XM_003434024.1	Spp1	1.44	5E-10	0.61	2E-21
XM_844217.2	Csad	1.44	2E-08	1.43	5E-10
XM_003432612.1	Trpm3	1.44	5E-09	1.83	3E-47

XM_850020.2	Il22ra1	1.43	8E-06	1.44	9E-07
XM_544900.1	Zbtb21	1.43	0.0004	1.53	3E-08
XM_542963.3	E2f1	1.43	1E-06	1.50	2E-09
XM_536720.4	Prdm16	1.42	0.0002	1.47	3E-05
XM_843994.2	Srebf2	1.42	9E-10	1.42	1E-14
XM_003432136.1	Sorbs2	1.42	1E-08	1.85	5E-37
XM_533524.3	Anxa1	0.70	8E-10	0.65	9E-31
XM_540914.3	Fads1	0.70	1E-05	0.64	6E-09
AY057077.1	Tpmt	0.70	8E-07	0.66	2E-09
XM_535987.3		0.70	8E-07	0.69	1E-08
XM_535330.3	Ti gl1	0.70	4E-11	0.54	2E-43
XM_543458.3	Tpst2	0.70	9E-05	0.70	3E-05
XM_536077.3	Tuba4a	0.70	6E-10	0.67	8E-18
XM_541532.4		0.69	1E-07	0.35	2E-51
XM_003432942.1		0.67	3E-08	0.68	1E-09
XM_545701.3	Rgs2	0.66	9E-10	0.68	7E-11
XM_535006.3	Col17a1	0.65	3E-15	0.67	3E-24
AY044905.1	Ptgs2	0.64	0.0002	0.52	1E-08
XM_003435547.1	Zfp185	0.64	7E-07	0.53	2E-11
AF314533.1	Il13ra2	0.64	9E-07	0.50	4E-14
AY455801.1		0.63	1E-13	0.67	5E-15
XM_003433320.1	Pid1	0.62	9E-08	0.31	6E-34
XM_536876.3	Baiap2l1	0.62	4E-18	0.70	1E-15
XM_536530.3	Aplp2	0.62	1E-19	0.64	7E-25
XM_850121.2	Tube1	0.62	1E-13	0.68	3E-11
XM_859070.2	Serpine2	0.61	3E-11	0.45	7E-29
XM_544107.1	Rrs1	0.61	3E-13	0.71	9E-10
XM_532542.3	Exo5	0.61	5E-17	0.64	2E-19
AF016649.2		0.61	2E-20	0.69	4E-18
XM_003435004.1		0.61	4E-14	0.70	2E-10
XM_544130.3	Jph1	0.60	2E-07	0.46	8E-14
NM_001003198.1	Pla2g7	0.60	2E-17	0.63	8E-22
XM_536702.3	Magoh	0.60	1E-18	0.70	5E-16
XM_544713.3	Lactb	0.60	2E-11	0.63	2E-12
XM_846683.2	1190005I06Rik	0.60	9E-18	0.68	4E-15
DQ975208.1	Ceacam1	0.58	1E-18	0.62	7E-21
XM_846177.2	9930012K11Rik	0.58	8E-14	0.66	3E-11
XM_541510.2	Fgf21	0.58	3E-17	1.43	3E-12
XM_536768.3	Gcsh	0.58	3E-16	0.66	3E-13
XM_539472.3	Gpnmb	0.58	5E-22	4.60	3E-224
XM_003433614.1		0.57	7E-20	0.65	7E-17
XM_846481.2	Itpa	0.56	2E-22	0.70	5E-16
NM_001197170.1	Col9a2	0.56	5E-18	0.68	6E-12
XM_003434948.1	Dsc3	0.56	1E-16	0.62	6E-14
XM_849226.3	Rgs11	0.55	4E-14	0.63	2E-12
NM_001197096.1	Ppp3ca	0.55	7E-21	0.70	2E-11
XM_536545.3	Sorl1	0.55	2E-18	0.59	3E-22
XM_538640.3	Tgfbi	0.55	9E-13	3.73	4E-58
XM_003432793.1		0.55	2E-11	0.70	1E-05
AY292464.1	LOC101056437	0.55	6E-18	1.51	2E-12

XM_544163.3	Cpne3	0.54	1E-19	0.61	4E-16
XM_545846.3	Aen	0.54	6E-26	0.59	1E-29
XM_848021.2	Ppif	0.54	2E-25	0.53	4E-45
XM_845057.2	Ralgps2	0.54	3E-20	0.64	3E-15
XM_534148.3	Lmo7	0.53	2E-25	0.66	2E-20
NM_001197095.1	Serpine1	0.53	2E-25	1.54	3E-15
XM_845599.2	Rgs17	0.53	5E-14	0.64	3E-09
XM_537128.3	Ddx59	0.53	3E-29	0.69	1E-19
XM_548004.3	Jag2	0.53	2E-25	0.58	3E-26
XM_003639810.1	Tex30	0.53	4E-19	0.57	3E-20
XM_003435145.1	Wdr89	0.52	8E-13	0.64	2E-07
XM_533382.3	Serpinb5	0.52	8E-23	0.57	7E-23
XM_853765.2	Myof	0.52	8E-31	0.61	9E-29
XM_539915.3	Atg9b	0.52	3E-22	0.57	7E-23
XM_539002.3	Col12a1	0.52	1E-19	2.93	5E-56
XM_537061.4	Dpyd	0.52	2E-32	0.53	1E-49
XM_845324.3	Lix1l	0.52	1E-17	0.69	2E-08
XM_538748.3	Tbc1d2	0.51	2E-27	0.66	9E-18
XM_534237.3	Tgfbr2	0.51	3E-38	0.58	4E-40
XM_003434708.1		0.51	4E-13	0.65	1E-07
XM_850178.2	R set2a	0.50	5E-30	0.65	4E-18
AF143503.1		0.50	5E-14	0.52	4E-15
XM_531763.3	Aldh1l2	0.50	1E-13	2.96	8E-63
XM_003433595.1	Sorbs1	0.50	4E-11	0.51	6E-11
XM_538032.3	Praf2	0.49	7E-38	0.68	6E-21
XM_539603.3	Gjb3	0.49	3E-27	0.61	5E-18
AY509607.1		0.49	5E-15	1.44	3E-07
AB031276.1	Mdm2	0.48	7E-40	0.55	5E-45
AF100705.1		0.48	4E-37	0.51	7E-53
AF394784.1	Dsg3	0.48	5E-38	0.66	1E-20
XM_545250.4	Il1rap	0.48	2E-21	0.70	9E-08
XM_846545.2	Daf2	0.48	3E-14	0.50	8E-14
XM_848524.2	Nqo1	0.48	6E-18	0.64	1E-09
XM_545529.3	Scrn3	0.48	8E-28	0.68	2E-11
XM_537579.3	Amz2	0.48	8E-41	0.63	3E-27
XM_548170.4		0.45	3E-18	0.67	2E-06
XM_003432574.2		0.45	3E-23	0.69	1E-07
AB011372.1	Slc12a4	0.44	1E-51	0.59	3E-39
XM_543863.3	9630033F20Rik	0.43	8E-31	0.49	1E-28
XM_537805.3	Olfm1	0.42	7E-32	0.53	6E-22
XM_540845.3	Fosl1	0.42	1E-24	0.60	2E-13
L28932.1		0.42	1E-41	0.69	3E-13
NM_001197022.1	Cd44	0.40	3E-53	0.68	5E-17
XM_845130.2		0.40	7E-44	0.48	1E-41
XM_003432338.1		0.40	1E-38	0.66	3E-09
XM_533922.3		0.38	6E-23	0.69	2E-18
XM_535151.3	9430020K01Rik	0.37	2E-25	0.50	8E-15
XM_548101.2	Krt16	0.36	2E-42	0.39	2E-48
XM_536441.3	Ccng1	0.36	3E-70	0.55	6E-42
XM_846790.2	Gch1	0.35	2E-37	0.59	4E-14

XM_543369.3	D hc10	0.35	2E-40	0.39	1E-40
XM_546036.3	Edil3	0.30	3E-42	0.59	6E-11
AJ830019.1		0.28	1E-101	0.42	2E-85
XM_532125.3	Cdkn1a	0.28	3E-107	0.42	2E-93
XM_003433852.1	Sytl1	0.26	3E-73	0.28	5E-92
XM_003431889.1		0.26	5E-52	0.57	9E-15
XM_003434922.2		0.24	1E-72	0.67	1E-11
XM_532641.3	Mgat4c	0.22	1E-40	0.40	6E-19
XM_542806.3	Esyt3	0.21	5E-50	0.39	2E-25
XM_846210.2	Slc10a6	0.20	4E-79	0.57	2E-14
DQ409210.1		0.20	2E-57	0.57	1E-10
AB066299.1		0.08	6E-88	0.20	4E-55
AF045016.1	Abcb1a	0.06	1E-110	0.17	4E-72

Supplementary Data-4.
Exon-4 of TP53 (ENSCAFP00000024579.3) from wild-type and p53-deficient MDCK clones.

wild-type
TCTTCGGAGCTGTGCCCAGCAGTGGATGAGCTGCTGCTCCCAGAGAGCGTCGTGAACTGGCTAGACGAAGACT
CAGATGATGCTCCCAGGATGCCAGCCACTTCTGCCCCCACAGCCCCTGGACCGGCCCCCTCCTGGCCCCTATCAT
CCTCTGTCCCTTCCCCGAAGACCTACCCTGGCACCTATGGGTTCCGTTTGGGGTTCCTGCATTCCGGGACAGCCA
AGTCTGTTACTTGGACG

p53 $^{-/-}$ Sequence 1
TCTTCGGAGCTGTGCCCAGCAGTGGATGAGCTGCTGCTCCCAGAGAGCGTCGTGAACTGGCTAGACGAAGACT
CAGATGATGCTCCCAGGATGCCAGCCACTTCTGCCCCCACAGCCCCTGGACCGGCCCCCTCCTGGCCCCTATCAT
CCTCTGTCCCTTCCCCGAATTCCTGCATTCCGGGACAGCCAAGTCTGTTACTTGGACG

p53 $^{-/-}$ Sequence 2
TCTTCGGAGCTGTGCCCAGCAGTGGATGAGCTGCTGCTCCCAGAGAGCGTCGTGAACTGGCTAGACGAAGACT
CAGATGATGCTCCCAGGATGCCAGCCACTTCTGCCCCCACAGCCCCTGGACCGGCCCCCTCCTGGCCCCTATCAT
CCTCTGTCCCTTCCCCGAAGACCTACCCTGGCACCTATGGGTTCCGTTTGGGGTTCCTGCATCCTGCATTCCGGGA
CAGCCAAGTCTGTTACTTGGACG

p53 $^{DB-1}$
TCTTCGGAGCTGTGCCCAGCAGTGGATGAGCTGCTGCTCCCAGAGAGCGTCGTGAACTGGCTAGACGAAGACT
CAGATGATGCTCCCAGGATGCCAGCCACTTCTGCCCCCACAGCCCCTGGACCGGCCCCCTCCTGGCCCCTATCAT
CCTCTGTCCCTTCCCCGAAGACCTACCCTGGCACCTATGGGTTCCGTTTGGGGTCCGGGACAGCCAAGTCTGTTA
CTTGGACG

p53 $^{DB-2}$ Sequence 1
TCTTCGGAGCTGTGCCCAGCAGTGGATGAGCTGCTGCTCCCAGAGAGCGTCGTGAACTGGCTAGACGAAGACT
CAGATGATGCTCCCAGGATGCCAGCCACTTCTGCCCCCACAGCCCCTGGACCGGCCCCCTCCTGGCCCCTATCAT
CCTCTGTCCCTTCCCCGAAGACCTACCCTGGCACCTATGGGTTCCTGCATTCCGGGACAGCCAAGTCTGTTACTTG
GACG

p53 $^{DB-2}$ Sequence 2
TCTTCGGAGCTGTGCCCAGCAGTGGATGAGCTGCTGCTCCCAGAGAGCGTCGTGAACTGGCTAGACGAAGACT
CAGATGATGCTCCCAGGATGCCAGCCACTTCTGCCCCCACAGCCCCTGGACCGGCCCCCTCCTGGCCCCTATCAT
CCTCTGTCCCTTCCCCGAAGACCTAAGACCTACCCTGGCACCTATGGGTTCACCTATGGGTTCCGTTTGGGGTTCC
TGCATTCCGGGACAGCCAAGTCTGTTACTTGGACG

p53 $^{DB-3}$ Sequence 1
TCTTCGGAGCTGTGCCCAGCAGTGGATGAGCTGCTGCTCCCAGAGAGCGTCGTGAACTGGCTAGACGAAGACT
CAGATGATGCTCCCAGGATGCCAGCCACTTCTGCCCCCACAGCCCCTGGACCGGCCCCCTCCTGGCCCCTATCAT
CCTCTGTCCCTTCCCCGAAGACCTACCCTGGCACCTATGGGTTCCGTTTGGGGTTCCTGCATTGGCACCTATGGG

TTCCGTTTGGGGTTCCTACCCTGGCACCTATGGGTTCCGTTTGGGGTTCCTGCATTGGCACCTATGGGTTCCGTTT
GGGGTTCCTGCATTCCGGGACAGCCAAGTCTGTTACTTGGACG

p53 **DB-3** Sequence 2

TCTTCGGAGCTGTGCCCAGCAGTGGATGAGCTGCTGCTCCCAGAGAGCGTCGTGAACTGGCTAGACGAAGACT
CAGATGATGCTCCCAGGATGCCAGCCACTTCTGCCCCCACAGCCCCTGGACCGGCCCCCTCCTGGCCCCTATCAT
CCTCTGTCCCTTCCCCGAAGACCTACCCTGGCACCTATGGGTTCCGTTTGGGGTTCCTGCAGACCTACCCTGGCA
CCTATGGGTTCCGTTTGGGGTTCCTGCATTCCGGGACAGCCAAGTCTGTTACTTGGACG