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Regulation of actomyosin contraction as a driving force of invasive lobular breast cancer

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General Discussion

Cancer is one of the major health problems faced by modern society. One in eight women is expected to develop breast cancer during their lifetime, making it the second most common cancer in women[1]. Over the past century, many advances have been made that improved the survival of patients. Nonetheless, breast cancer remains responsible for most cancer related deaths in women[1]. We have learnt that cancer is very heterogeneous disease and that each tumor is unique in its genetic makeup. This is not to say that there is no overlap between tumors. Many tumors share certain genetic alterations and/or phenotypes allowing classification into different subtypes. Invasive lobular carcinoma (ILC) is a distinct morphological subtype of breast cancer representing 8-14% of all breast cancer cases. In ILC, loosely attached tumor cells typically invade the surrounding stroma in single files. The main hallmark of ILC is the loss of the intercellular adhesion protein E-cadherin (encoded by *CDH1*). In this thesis we delved deeper into the development of ILC and the molecular mechanisms involved in this process. We used genetically engineered mouse models and insertional mutagenesis to identify novel drivers of ILC. We then focused on the mechanisms by which these novel drivers contribute to ILC initiation and progression. Finally, we used insertional mutagenesis to discover mechanisms of resistance to FGFR inhibitors. In this chapter, I will discuss some of the approaches used in the previous chapters and put the main findings of this thesis into context.

Insertional mutagenesis as a tool to identify novel cancer drivers and resistance mechanisms

The transformation of normal cells into cancer cells by the accumulation of (epi)genetic alterations is an important hallmark in tumorigenesis. Cancer cells frequently have large numbers of changes in numerous genes making it difficult to ascertain which mutations are responsible for tumor initiation and progression. In this thesis, we have employed insertional mutagenesis (IM) to identify genes involved in both the development of cancer (**Chapter 2**) and the acquisition of resistance to anti-cancer therapeutics (**Chapter 5**).

IM has two main advantages over other mutagenic approaches. First, targeted sequencing allows for rapid and robust retrieval of transposons insertions enabling quick identification of the affected genes. Second, IM can be used to discover both tumor suppressor genes and oncogenes in a single setting when the transposon system contains both a promoter sequence and transcriptional termination elements. A particular strength

of IM screens is the ability to identify driver genes that are not readily apparent in human sequencing data. Examples of these are genes that are undergoing alternative splicing or genes located on amplified/deleted chromosomal regions encompassing multiple genes making it unclear which gene(s) are causal to tumorigenesis. This strength is highlighted in **Chapter 2**, where we showed that three drivers of ILC in mice, MYPT2, ASPP2 and MYH9, are also frequently mutated by chromosomal aberrations in human ILCs.

While IM is a useful tool to identify drivers of tumorigenesis and drug resistance, it also has several limitations that one should be aware of. An important limitation of IM systems is that they are inherently incapable of generating point mutations. IM therefore cannot recapitulate all potential aberrations found in human tumors. Complementation of IM screens with genome-wide sequencing approaches could identify somatic SNVs in tumors, but their frequency is highly dependent on the model system used. Another limitation of the available IM systems is their bias[2]. IM systems often have different integration preferences and integration rates[3,4]. The composition of the transposon is another important factor that can introduce bias by affecting cell type specificity. For example, the MSCV promoter in the Sleeping Beauty (SB) T2/Onc2 transposon is more active in hematopoietic cells than in other cell types, while the CAG promoter in the T2/Onc3 transposon is expressed in multiple cell types[5,6]. The T2/Onc2 transposon system was used in **Chapters 2** and **5** which might explain why relatively few oncogenes were identified in our screen compared to other SB-based insertional mutagenesis screens[7,8].

Overall, the work presented in this thesis and previous work from others shows that IM is a powerful tool to discover genes contributing to tumor development and drug resistance. IM can work complementary with human tumor characterization studies to distinguish relevant driver genes from irrelevant passengers.

Consequences of E-cadherin loss in mammary epithelial cells

For the last 15 years, it was believed that upon loss of E-cadherin expression, mammary epithelial cells (MECs) are extruded into the lumen where they undergo apoptosis[9–11]. This theory explained why mice with mammary gland-specific loss of E-cadherin did not immediately develop tumors and suggested that additional mutations were required to induce ILC. What

this theory did not adequately explain was the increased susceptibility of *Wcre;Cdh1*$^{F/F}$ mice to tumor development compared to *Wcre;Cdh1*$^{F/+}$ mice (observed in **Chapter 4**). In **Chapter 3,** we show that loss of E-cadherin in murine MECs (MMECs) causes not only extrusion into the lumen but also extrusion towards the basement membrane and the fibrous stroma. Whereas luminal extrusion results in apoptosis, we never observed any apoptosis in the basally extruded cells. The experiments done in **Chapter 3** suggest that the basally extruded E-cadherin-deficient MMECs do not go into apoptosis because they can adhere to the basement membrane component laminin. Laminin is present in the basement membrane directly surrounding the mammary ducts and was also found around the basally extruded MMECs. In the fibrous stroma, the basally extruded MMECs can persist and form clusters of cells. These clusters typically do not increase in size over time explaining the lack of immediate tumor formation in *Wcre;Cdh1*$^{F/F}$ mice. However, the fact that these basally extruded MMECs persist could allow these cells to gain additional mutations over time that enable tumorigenesis, potentially explaining the late onset of mammary tumors in a subset of the *Wcre;Cdh1*$^{F/F}$ mice (**Chapters 2** and **4**). It would also explain the increased predisposition of female *CDH1* mutation carriers to ILC development[12,13].

To validate this finding in a more clinically relevant setting, it would be interesting to look at healthy breast tissue of *CDH1* mutation carriers to see if basally extruded E-cadherin-deficient MECs are present. It should however be noted that the *CDH1* germline mutations in mutation carriers are heterozygous, whereas tumors in these patients are hallmarked by loss of the wild-type (WT) allele. Hence, the frequency of E-cadherin loss is likely to be much lower than in our *Wcre;Cdh1*$^{F/F}$ mice, making it more difficult to detect these cells. Alternatively, it might be possible that loss of E-cadherin is not the first step of ILC initiation, but rather occurs after different mutations have been acquired that allow escape from the mammary epithelium and growth on fibrillar collagen in the mammary stroma. However, if this is the case, one would expect that portions of the tumor are E-cadherin proficient, which only happens in a minority of ILCs. If basally extruded E-cadherin-deficient MMECs are indeed present in *CDH1* mutation carriers, it would be interesting to see if these cells could be cleared as a strategy to reduce or even prevent ILC development in these women. A potential strategy might be to target the interaction with laminin, since our findings suggest that E-cadherin-deficient MECs rely on the interaction with laminin for their survival in the fibrous stroma.

One important question that we were unable to address is what percentage of the MMECs that lose E-cadherin expression undergoes luminal- versus basal extrusion. The reason for this is that the luminally extruded MMECs undergo apoptosis and are rapidly cleared. It would be interesting to use intravital imaging to observe this process in real time. For this purpose, it would be essential to use a mouse model where E-cadherin is flanked by loxP sites and coupled to a fluorophore to determine the exact moment when E-cadherin expression is lost.

In summary, we have found that mammary epithelial cells undergo basal extrusion upon loss of E-cadherin and persist in the fibrous stroma. This finding would also corroborate the susceptibility of *CDH1* mutation carriers and the decrease in tumor latency observed when E-cadherin loss is combined with other ILC drivers. This finding also supports the theory that loss of E-cadherin is first event in ILC development rather than a mutation acquired in later stages.

Actomyosin contractility as a barrier for ILC development

The actin cytoskeleton is an essential component of every cell that is not only responsible for the shape of the cell but also closely involved in signal transduction. Contraction of actin fibers is required for a host of cellular processes ranging from migration to cell division[14]. Like most cellular processes, actomyosin contractility is strictly regulated and the amount of contractility differs greatly from one cell type to another. *In situ* luminal mammary epithelial cells typically have low actomyosin contractility compared to the surrounding myoepithelial cells, as demonstrated by low levels of myosin light chain phosphorylation (**Chapter 3 Fig 2e, f**).

In the IM screen reported in **Chapter 2**, we identified MYPT1/2, ASPP2 and MYH9 as novel ILC drivers, of which only ASPP2 was not yet known to be involved in actomyosin contractility. Further analysis revealed that the truncation variants of MYPT1 (**Chapter 3**) and ASPP2 (**Chapter 4**) identified in the IM screen both promote actomyosin relaxation by dephosphorylating myosin light chains, while reduction of MYH9 directly reduces actomyosin contraction. This was a surprising finding since high levels of contractility have been considered to be a protumorigenic trait of tumor cells[15–17]. Additionally, luminal MMECs normally have low levels of contractility, making it implausible that further reduction would be beneficial to these cells. However, the basally extruded E-cadherin-deficient MMECs that

persist in the fibrous stroma often displayed relatively high levels of myosin light chain (MLC) phosphorylation and increased membrane blebbing, a process which is caused by high levels of actomyosin contractility[18,19].

The increased actomyosin contractility in basally extruded MMECs is likely caused by both cell-intrinsic and -extrinsic factors. Engagement of E-cadherin has been shown to result in reduced RhoA signaling by activation of p190RhoGAP[20,21]. In addition, loss of E-cadherin leads to increased cytoplasmic and nuclear localization of p120[22]. In the absence of E-cadherin, p120 has been found to increase RhoA activity by inhibiting myosin phosphatase Rho-interacting Protein (MRIP)[23]. In addition to the effect of E-cadherin loss on RhoA activity, the extracellular environment that E-cadherin-deficient MMECs encounter upon basal extrusion also influences actomyosin contractility. It has been shown that integrin engagement to fibrillar collagen (Collagen 1) increases RhoA activity[24]. The extracellular matrix surrounding the basally extruded MMECs (and ILCs) is rich in fibrillar collagen, explaining the increase in actomyosin contractility. However, not all basally extruded MMECS displayed higher pMLC levels, indicating that some cells do not have increased actomyosin contractility. This is likely due to the presence of laminin around some of the clustered cells (**Chapter 3 Fig 6a**). Engagement of integrins to laminin reduces RhoA activity and thereby lowers actomyosin contractility[24]. The presence of laminin around the basally extruded clusters likely also enables the survival of these cells in the fibrous stroma surrounding the mammary ducts. Our *in vitro* experiments in **Chapter 3** support this theory as E-cadherin-deficient MMECs were able to adhere and survive on laminin 332-coated matrix but not on a collagen 1 matrix. It remains unclear whether laminin is produced by the E-cadherin-deficient MMECs, by other cell types such as myoepithelial cells, or a combination thereof.

Our results show that E-cadherin-deficient MMECs benefit from actomyosin relaxation but only to a certain degree (**Chapter 3 Fig 4**). Too much relaxation is not tolerated and prevents growth of E-cadherin-deficient MMECs. This is not surprising since actomyosin contractility is required for a number of cellular processes including cytokinesis[25,26]. The notion that expression of t-ASPP2 (and likely also t-MYPT1) does not completely inhibit actomyosin contraction is highlighted by pMLC phosphorylation at the cleavage furrow of a t-ASPP2 expressing E-cadherin-deficient cell undergoing cytokinesis (**Chapter 4 Supplementary Fig 6**). The optimal amount of actomyosin contractility is likely different from one cell to another, depending on their

environment, transcriptional program and the presence of oncogenic mutations.

Our finding that high levels of actomyosin contractility form a barrier for ILC formation does not mean that E-cadherin-deficient MMECs cannot progress into ILCs without reducing their actomyosin contractility. Additional mutations in other oncogenes/tumor suppressor genes (e.g. p53) could allow E-cadherin-deficient MMECs to tolerate the negative consequences of high levels of actomyosin contractility[10,27]. Those tumor cells can then actually benefit from high levels of actomyosin contractility as is evident from the dependency on ROCK signaling in ILCs induced by combined loss of E-cadherin and p53[23].

Overall, we have shown that increased actomyosin contractility in basally extruded E-cadherin-deficient MMECs forms a critical barrier to ILC development. There appear to be multiple ways to overcome this barrier and progress into ILC.

ASPP2 and actomyosin contractility

Of the four hits identified in the IM screen described in **Chapter 2**, only ASPP2 had not previously been associated with actomyosin contractility or the regulation thereof. The mutual exclusivity of the transposon insertions in these four genes suggests that they either have functionally redundant roles, are synthetic lethal or both. The results in **Chapters 2-4** show that truncated ASPP2 (t-ASPP2) leads to dephosphorylation of MLC by interacting with PP1 in a manner similar to truncated MYPT1 (t-MYPT1). The amount of MLC dephosphorylation induced by t-ASPP2 is also equal to that induced by t-MYPT1, favoring a direct interaction. However, we still lack direct evidence that a complex comprising t-ASPP2 and PP1 can directly dephosphorylate MLC.

The second remaining question is whether wild-type ASPP2 also causes (direct) dephosphorylation of MLC, and if so, what biological function this serves. A potential effect of wild-type ASPP2 on MLC phosphorylation will likely be less than t-ASPP2 since t-ASPP2 constitutes a hyperactive mutant that lacks the majority of the proline rich domain which inhibits ASPP2 function[28]. In addition, ASPP2 has multiple functions some of which are independent of its interaction with PP1 and might therefore compete with PP1 binding and/or function. Knockout experiments have shown that ASPP2 plays a role in the maintenance of epithelial identity through repression

of ZEB1[29]. Since epithelial cells tend to have lower levels of actomyosin contractility compared to mesenchymal cells, it is possible that ASPP2-mediated actomyosin relaxation plays a role in maintaining this phenotype. The ideal way to study the potential role of ASPP2 in regulating actomyosin contractility would be to generate a mutant that no longer interacts with MLC while retaining all other functions. The generation of this ASPP2 mutant is challenging because it remains unclear which residues are responsible for the interaction with MLC. It is likely, however, that the interacting amino acids are located somewhere in the ankyrin repeat domain, similar to MYPT1[30].

Clinical evidence for actomyosin relaxation in ILC

Out of the four mutually exclusive driver genes identified in IM screen, three genes are frequently altered in human ILC (**Chapter 2 Fig 4**). Both *MYPT2* and *ASPP2* are located on the chromosome 1q, which is frequently amplified in ILC. The transposon insertions in *Mypt1/2* and *Aspp2* resulted in expression of dominant-active truncation variants while the amplifications found in human ILCs presumably lead to increased expression of WT MYPT2 and ASPP2. In **Chapter 3**, we show that overexpression of WT MYPT1 in E-cadherin-deficient MMECs is also sufficient to induce ILC. The ILCs induced by overexpression of WT MYPT1 were smaller than those induced by t-MYPT1, likely because WT MYPT1 can be inactivated by ROCK whereas t-MYPT1 cannot. Therefore, it is possible that it would be advantageous to overexpress both, MYPT2 and ASPP2 rather than the mutual exclusive expression of t-MYPT1 and t-ASPP2 observed in the IM screen.

In addition to co-amplification of WT MYPT2 and ASPP2, human breast cancers have also been reported to express truncation variants of ASPP2. Van Hook et al. reported overexpression of an ASPP2 truncation variant that utilizes an alternative start codon in exon 8 of *ASPP2*[31]. While this truncation variant is not exactly the same as the truncation variant described in **Chapters 2 and 4**, both ASPP2 variants lack the N-terminus that may negatively regulate ASPP2 activity. The ASPP2 variant discovered by van Hook et al. might therefore also induces actomyosin relaxation. In addition, it is possible that the reason this alternative variant of ASPP2 is overexpressed in breast cancer is because of the 1q amplification described before. It remains to be determined whether the truncation variant identified by van Hook et al. is expressed in lobular breast cancers.

MYH9 is located on chromosome 22q. Shallow deletions of 22q are frequently observed in ILC patients and correlate with decreased RNA expression of *MYH9* (**Chapter 2 Fig 4**). Decreased expression of MYH9 is in line with what we observed in the tumors generated by *in vivo* CRISPR/Cas9 mediated gene editing of *Myh9* in E-cadherin-deficient MMECs. These tumors invariably harbored one in-frame and one out-of-frame deletion in *Myh9*, supporting the notion that MYH9 is a haploinsufficient tumor suppressor.

Also other mutations in human ILC might result in reduced actomyosin contractility. For example, RhoA mutations are more often found in lobular breast cancer (3.3%) compared to ductal breast cancer (0.5%)[32,33]. The majority of the observed alterations are $RhoA^{G17V/E/A}$ missense mutations, which have been shown to act dominant-negatively as they are incapable of binding to ROCK and thus prevent its activation, resulting in reduced actomyosin contractility[34,35].

While the 1q amplifications and the shallow deletions of 22q provide evidence for a role of MYPT2, ASPP2 and MYH9 in ILC development, they are not conclusive proof that the amplifications/shallow deletions of these genes are causal to the development of human ILC. It remains possible that other genes located on these aberrated chromosomal regions play a role in tumorigenesis. There are two possible options to validate that MYPT2, ASPP2 and MYH9 are causal to human ILC development. First, one could systematically test all genes located on the 1q/22q chromosomal regions to determine if *MYPT2*, *ASPP2* and *MYH9* are the only genes capable of inducing ILC. In the past, such an approach would have been a herculean task but with the advent of somatic breast cancer modeling via intraductal injections and CRISPR/Cas9-mediated gene editing this is now feasible[36,37]. The second approach would be to make use of human ILC PDX models and/or cell lines that harbor an 1q amplification or 22q shallow deletion. Knockout or knockdown of MYPT2/ASPP2 or overexpression of MYH9 in these models would allow us to determine if they are dependent on these driver events. At first glance, the latter approach might seem like the more clinically relevant approach. However, it has proven difficult to generate cell lines and PDX models from ILCs, and those that were successful have typically been derived from more aggressive tumors or metastases. In addition, we have primarily investigated the role of MYPT2, ASPP2 and MYH9 in tumor initiation and have not evaluated whether ILCs remain dependent on actomyosin relaxation when they are already established.

A potential role for actomyosin relaxation in the development of other tumor types has not been extensively investigated. The only direct evidence that actomyosin relaxation can be beneficial was observed in diffuse gastric cancer (DGC), a cancer type that is also known for the loss of E-cadherin[35,38]. DGCs frequently harbor RhoA mutations that inhibit ROCK, which in turn inhibits MLC phosphorylation. However, the most common RhoA missense mutation in DGC, Y42C has also been shown to increase ROCK binding and result in increased actin stress fiber formation in 3T3 fibroblasts[39,40]. It is possible that the discrepancy between these findings might be in part due to the difference in cell types used. Fibroblasts have higher levels of contractility and might therefore respond differently to the expression of RhoA mutants than epithelial cells, which generally have lower levels of contractility. The *RhoA* mutations in DGC are mutually exclusive with fusions between *CLDN18* and the RhoGAPs *GAP6* and *GAP26*, which occur in around 15% of all DGC patients[41]. These fusions have been reported to result in RhoA inhibition and decreased actomyosin contractility[35].

Overall, it appears that there is a subset of human ILCs that harbor mutations that reduce actomyosin contractility. It remains unclear, however, whether these alterations are causal to ILC development. Future efforts should focus on trying to identify driver genes located on chromosomal regions frequently affected by copy number alterations in multiple model systems including patient-derived models. It will also be interesting to investigate the tumorigenic potential of the RHOA-G17V/E/A mutations in ILC.

Actomyosin contractility in tumor progression

In **Chapters 3** and **4** of this thesis, we focused on the effects of actomyosin relaxation on tumor initiation. In **Chapter 4**, we also investigated the progression of tumors driven by combined loss of E-cadherin and actomyosin relaxation. It became apparent that tumors initiated by combined loss of E-cadherin and partial actomyosin relaxation grow and progress very slowly. The tumor cells are locally very invasive but show low levels of proliferation. The growth of these tumors is likely purely driven by the proliferative effects caused by loss of E-cadherin[42–44], which are rather subtle in comparison to other oncogenic driver events like ERBB2 activation. Importantly, also classic ILCs in patients typically display low proliferation rates[45]. The models described in this thesis might therefore more accurately reflect some aspects of slow-growing classic ILCs observed in the clinic.

Given the evidence pointing towards a pro-tumorigenic role of actomyosin contractility, one might expect that actomyosin relaxation would actually slow down tumor progression and or growth. However, we observed that mice in which mammary-specific loss of E-cadherin and PTEN was combined with t-MYPT1 expression develop larger tumors than mice with mammary-specific loss of E-cadherin and PTEN alone. It is possible that the increased tumor size is due to the earlier tumor initiation, but this experiment at least indicates that actomyosin relaxation does not hamper tumor growth in this setting. Nevertheless, it is still possible that actomyosin relaxation decreases the metastatic potential of ILCs driven by loss of E-cadherin and PTEN.

Actomyosin contractility as a therapeutic target

The discovery of actomyosin contractility as a pro-tumorigenic trait has sparked the development of multiple therapeutic agents that target proteins involved in this pathway[33,46]. Most of the developed compounds are ROCK inhibitors, which have shown promising activity in preclinical models[47]. Multiple ROCK inhibitors have entered clinical trials and have proven to be nontoxic, and several have been approved for the treatment of cerebral vasospasms, pulmonary hypertension and glaucoma[46,48]. However, the clinical efficacy of ROCK inhibitors as anti-cancer drugs has proven to be disappointing, and none have been approved for the treatment of cancer as of yet. Current efforts are focused on the development of more specific inhibitors and combinations of ROCK inhibitors with other compounds.

While ROCK inhibitors seem to be able to decrease tumor invasion and growth they are not capable of inducing tumor regression[49]. Much of the current research with ROCK inhibitors is therefore focused on preventing invasion and metastatic disease. However, given the evidence that dissemination can already start relatively early on, it might be unwise to target invasion if the tumor cells have already arrived at metastatic sites at the time of treatment[50,51]. There is also evidence that ROCK inhibition can affect the immune system and thereby increase anti-tumor immunity[52]. However, It is currently unclear whether modulation of the immune system via ROCK inhibition will be exclusively anti-tumorigenic since ROCK activity has also been shown to be important for immune cell activation[53,54].

The results described in **Chapter 3** and **4** of this thesis and published data from other groups suggest that ROCK inhibition can also increase cell

survival and in some conditions even promote tumor development[55–59]. We showed that the survival-promoting effect of ROCK inhibition is at least in part caused by reducing actomyosin contractility. Furthermore, we showed that the pro-tumorigenic effect of ROCK inhibition is dose dependent as high levels of ROCK inhibition are not compatible with survival of E-cadherin-deficient MMECs. This suggests that complete actomyosin relaxation is not tolerated, which is in line with previous findings[25]. However, we cannot rule out that off target effects are responsible for the reduced growth of E-cadherin-deficient MMECs in the presence of high concentrations of the ROCK inhibitors. One could argue that tumors harboring mutations that cause actomyosin relaxation might be more sensitive to ROCK inhibitors since they already have low levels of contractility and cannot survive when actomyosin contractility is further inhibited. However, in our *in vitro* assays we see only a minor reduction in growth when ROCK inhibition is combined with expression of t-MYPT1 or t-ASPP2, suggesting this is not the case. An explanation for this might be that since both truncation variants are dominant active, ROCK inhibition only has a limited effect on actomyosin contractility under these conditions. Blebbistatin might therefore be much more detrimental to cells that express t-MYPT1 or t-ASPP2 because it inhibits actomyosin contractility directly.

Overall, it has become clear that it is critical to determine which tumor (sub)types are dependent on high levels of ROCK activity/actomyosin contractility and what effects ROCK inhibition might have on the tumor environment.

Concluding remarks

The transformation of normal cells into tumor cells is a multi-step process that depends on multiple cell-intrinsic and -extrinsic factors including the cell of origin, sequence of events and surrounding environment. In the studies presented in this thesis, we have focused primarily on the requirements and mechanisms involved in the development of invasive lobular breast cancer. We have shown that loss of E-cadherin in luminal MMECs not necessarily results in their demise but also induces their extrusion to the fibrous stroma where they can persist for prolonged periods. Basally extruded E-cadherin-deficient MMECs do not invade the mammary fat pad because they are incapable of adhering and surviving on fibrillar collagen due to aberrant levels of actomyosin contractility. Rebalancing of actomyosin contractility in E-cadherin-deficient MMECs by increasing myosin phosphatase activity,

reducing MYH9 levels or inhibiting ROCK activity promotes ILC formation in mice. This was a surprising finding since high levels of actomyosin contractility are typically associated with increased malignancy. While rebalancing of actomyosin contractility in E-cadherin-deficient MMECs enables rapid ILC formation, the growth and progression of these tumors is very slow. It is therefore important to note that actomyosin relaxation in this setting promotes tumor initiation rather than tumor growth. While there are strong indications that a subset of human ILCs harbor mutations that induce actomyosin relaxation, their dependency on these mutations remains to be evaluated. It also remains to be investigated whether tumors driven by actomyosin relaxation harbor specific vulnerabilities that can be exploited therapeutically. The mouse models described in this thesis can serve as powerful preclinical tools for testing novel therapeutic strategies and for identifying potential mechanisms of drug resistance.

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