Physiological roles of the oncoprotein c-Myc in mouse mammary gland development

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**SUMMARY**

c-Myc, a transcription factor that affects a large number of target genes, is one of the most frequently deregulated oncoproteins in human cancer. The wide-ranging biological functions of c-Myc include regulation of the cell cycle, differentiation, metabolism and growth, protein synthesis and chromatin modifications. For more than 25 years, many labs have focused their research on the mechanisms and pathways that are affected by c-Myc in normal development and in cancer, including breast cancer. However, relatively little was known about the normal physiological impact of c-Myc on the mammary gland before this study.

To address this open question and uncover the roles of c-Myc during mouse mammary gland development, we used a mammary gland specific conditional knockout approach, as the full body knockout of c-Myc is embryonic lethal. For this, we crossed $c\text{-}myc^{fl/fl}$ mice to mice heterozygous for the WAPiCre transgene (Cre recombinase under the control of the Whey acidic protein promoter). This resulted in c-Myc loss exclusively in the milk-producing, luminal alveolar epithelial cells starting in mid-pregnancy. Three major phenotypes were identified in the glands of mutant mice. First, we observed that pups nursed by c-Myc mutant mothers grew slower compared to pups nursed by wild type mothers. While milk composition was comparable between wild type and mutant animals, we found that milk production was reduced in c-Myc mutant glands. Electron microscopy revealed that there were less secretory vesicles budding from the endoplasmic reticulum in lactating mutant cells, suggesting a decreased protein synthesis. By performing polysome fractionation experiments we showed that translational efficiency was generally decreased in mutant glands. In addition, we found that levels of ribosomal proteins and rRNA were lower in mutant glands. Interestingly, analyzing mRNA distribution along the polysome gradient demonstrated that mRNAs whose protein products are involved in milk synthesis were specifically affected while mRNAs of house keeping genes were generally unchanged. Our second major finding was that in a second round of pregnancy, c-Myc-deficient cells displayed a slower proliferation early
during pregnancy. The delayed proliferative response led to delayed but not blocked alveologenesis. Finally, the third major observation in c-Myc-deficient glands is related to progenitor cells. In mammary transplantation assays, epithelium from mutant glands showed a reduced ability to repopulate the glands of female recipients compared to epithelium from wild type glands, suggesting a role for c-Myc during this process.

To summarize, we show here for the first time that c-Myc plays multiple roles in the mouse mammary gland. Conditional loss of c-Myc caused delayed proliferation and differentiation during pregnancy. During lactation, milk production and translation were decreased in mutant glands. Finally, results from transplantation studies suggest a role for c-Myc in progenitor cell proliferation and/or survival.
INTRODUCTION

I. Mammary gland development and breast cancer

The mammary gland is a unique organ of female mammals, which enables them to supply their offspring with all essential nutrients via the milk produced in the gland. Two features have made the mouse mammary gland a very attractive model organ for biological research: first, most of its development occurs after birth and second, with every round of pregnancy the organ goes through repeated cycles of proliferation, differentiation and apoptosis. The tremendous changes and differentiation that convert the organ into a milk synthesizing machine have been subject to intense investigation for many years. By deciphering the tightly regulated hormone signaling networks, researchers wish to gain insights especially into breast cancer susceptibility, development and metastasis. Breast cancer is the second cancer worldwide in term of incidence, with 1.29 million out of 12.4 million new cancer cases reported in 2008 (World Cancer Report 2008). While large effort has been made in the twentieth century and multiple treatments are available, there is, depending on the type of breast cancer, much room for improvement. For better understanding of the disease, it is indispensable to know the mechanisms in normal breast development, starting from the model organism mouse and finding the parallels in human breast.

1.1 development: brief overview

The mammary gland consists of two main compartments: the epithelium, forming a bi-layered ductal network of luminal and basal cells, and this is embedded in the stroma or fat pad, which consists mostly of adipocytes and stromal cells, and also contains blood vessels and cells of the immune system. In addition, each fat pad is connected to the outside via the nipple, and harbors (at least) one major lymphnode (Fig. 1). Development of the gland starts in the embryo as an appendix of the ventral skin around embryonic day 10, where the milk line defines the position of the later
glands. Various well known signaling molecules regulate the developmental stages until birth, at which the gland already consists of a rudimentary ductal tree within a stromal fat pad (for detailed review of signaling in embryonic development see Robinson, 2007; Watson and Khaled, 2008). At the start of puberty, at around 3 weeks of age, hormones secreted by the ovaries induce the appearance of highly proliferative structures at the tips of the ducts, called terminal end buds (TEB), which start to invade the fat pad (Richert et al., 2000). Ductal elongation continues until approximately 10-12 weeks of age, when TEBs disappear but secondary and tertiary side branches still continue to form in the mature gland during estrus cycles.

![Figure 1: Mammary gland development after birth. Pubertal development and pregnancy cycle. Age or duration of stage are indicated. TEB: terminal end bud, LN: lymphnode.](image)

When pregnancy occurs, a tremendous proliferation and differentiation cascade is initiated, driven by various hormones. After 19-21 days of pregnancy, secretory activation occurs at parturition and copious amounts of milk are produced in the secretory units, the alveoli. Lactation continues for approximately 3 weeks, when
pups are weaned and milk stasis induces the complex apoptosis and remodeling phase called involution. The fully involuted (after 21 days) gland morphologically resembles a virgin-like stage, ready for a next round of pregnancy. The important steps during this development are discussed in the following sections in more detail.

1.2 puberty and estrus cycle

After birth, the rudimentary mammary gland grows in parallel to over-all body growth (allometric growth) for the first weeks, until levels of the steroid hormones estrogen and progesterone rise in puberty and TEBs are formed. The TEBs are highly proliferative and migratory structures, which penetrate the fat pad during ductal elongation (Ball, 1998). The TEBs consist of two major cell types (Fig. 2): one single layer of cap cells, that differentiate into myoepithelial cells in the subtending duct, and multiple layers of body cells, which are highly proliferative at the tip of the TEB but apoptotic in the inside, thus forming a lumen (Howlin et al., 2006).

Figure 2: Schematic diagram of a TEB. The characteristic features of the TEB and the subtending duct within the stromal surrounding.
Primary ducts are generated by bifurcation of TEBs and, together with the lateral-sprouting secondary side-branches, they fill the entire fat pad with a ductal network (Sternlicht, 2006). The number and size of lateral buds increase with every estrus cycle. In mature virgins, during the 4 day estrus cycle, repeated phases of proliferation, mild differentiation and apoptosis take place (Andres and Strange, 1999; Richert et al., 2000). Proliferation is high in late proestrus and estrus, and lateral buds develop into tertiary branches or alveolar buds. During estrus, transient expression of differentiation markers (i.e. milk proteins) can be detected (Robinson et al., 1995). In contrast, regionally restricted apoptosis is prominent during diestrus, where whole alveolar structures disappear.

Various genetic mouse models have identified hormones, growth factors (GFs), transcription factors (TFs) etc. involved in pubertal mammary gland development (reviewed in Howlin et al., 2006), of which the 3 main hormones are discussed below. The two ovarian hormones, estrogen (E) and progesterone (P), were shown to be mostly responsible for ductal elongation and tertiary side branching/alveolar expansion, respectively (Hennighausen and Robinson, 2005). Of the two estrogen receptor (ER) isoforms, ER\(\alpha\) and ER\(\beta\), epithelial ER\(\alpha\) was shown to be required for ductal outgrowth, whereas stromal ER\(\alpha\) was not required for wild type (WT) epithelium to develop normally (Mallepell et al., 2006). Epithelial ER\(\alpha\) acts in a paracrine fashion and by inducing expression of amphiregulin, the major pubertal EGFR ligand, neighboring cells are stimulated to proliferate (Ciarloni et al., 2007). Progesterone receptor (PR) also exists in two isoforms, PR-A and PR-B, of which PR-B is reported to be required for normal side-branching while PR-A seems to be dispensable (Conneely et al., 2001). Similarly as ER, PR signaling is also thought to be paracrine, and Wnt4 was found to be a potential PR target that induces branching morphogenesis in vivo (Brisken et al., 2000). The third major influence in the pubertal mammary gland is the pituitary growth hormone (GH) and its receptor (GHR). Knockout (KO) studies have shown that unlike ER and PR, GHR is required in the mammary stroma for successful ductal outgrowth and side-branching (Gallego et al., 2001). GHR also functions in a paracrine way, by increasing local IGF-1 (insulin-like growth factor 1) expression in the mammary gland which, in synergy
Introduction: Mammary gland

with estrogen, induces proliferation in neighboring epithelial cells (Kleinberg, 1997; for recent review of paracrine factors in the mammary gland see Rosen, 2009). Also prolactin (Prl) and its receptor (PrlR) were suggested to play a role during puberty, as both $Prl^{-/-}$ and $PrlR^{-/-}$ mice showed severe defects in ductal side branching and TEB morphogenesis while prepubertal growth was unaffected (Horseman et al., 1997; Ormandy et al., 1997). Transplantation experiments with $PrlR^{-/-}$ tissue into WT hosts demonstrated that $PrlR^{-/-}$ epithelium is able to develop normally until pregnancy, showing that the effect observed in a full body KO for PrlR is more likely due to requirement of endocrine Prl signaling (Brisken et al., 1999).

1.3 pregnancy and lactation

During approx. 19 days of pregnancy, various stimuli induce the massive changes that convert the mammary gland into a ‘milk factory’. The development of functional alvoeli, alveolar morphogenesis, is a combined process of proliferation and differentiation. Ductal proliferation peaks around day 3 of pregnancy and is low afterwards. Alveolar proliferation begins to increase around day 5 of pregnancy and peaks around day 7 and day 12 (Borst and Mahoney, 1982). Differentiation, also called lactogenesis, starts around mid-pregnancy and can be divided into lactogenesis I (secretory initiation) and lactogenesis II (secretory activation shortly before parturition) (Neville et al., 2002). The involvement of multiple hormonal and GF inputs as well as the tremendous changes in gene expression, signaling and metabolism are the subject in the following sections.

1.3.1 start of pregnancy: role of prolactin signaling

At the very beginning of pregnancy, initiated by mating activity, Prl is secreted from the pituitary, which is the key player in alveolar morphogenesis, together with P (Neville et al., 2002). The synergistic action of the two hormones is supported by a feedback loop, as Prl stimulates sustained secretion of ovarian P (and also E) and P in turn induces expression of PrlR (Oakes et al., 2006). The importance of Prl signaling during early pregnancy was demonstrated in heterozygous $PrlR^{+/-}$ mice where epithelial cell proliferation was severely impaired leading to lactation failure.
Interestingly, this haploinsufficiency could be partially overcome in subsequent pregnancies, which was explained by a requirement for certain levels of PrlR that could only be acquired in a second pregnancy (Ormandy et al., 1997). Transplantation experiments of PrlR<sup>−/−</sup> cells into WT hosts revealed that the pregnancy related function of Prl signaling was autonomous to the epithelial compartment (Brisken et al., 1999).

Signaling via Prl and PrlR is mainly mediated via the Jak2-Stat5 pathway (Fig. 3, for detailed reviews see Oakes et al., 2006; Oakes et al., 2008). Stat5, originally called mammary gland factor, activates transcription of multiple targets involved in alveolar morphogenesis, including proliferation, polarity, cell-cell and cell-stroma interaction and finally milk protein expression in lactation (Wakao et al., 1995).

**Figure 3: Prolactin signaling during pregnancy.** Grey circles indicate receptor specific ligands, i.e. P for PR, Prl for PrlR, RankL for Rank, e.g. heregulin for ErbB4 and e.g. laminin for β1-integrin. Abbreviations and explanations are given in the text.

Signaling via Stat5 is regulated by various positive and negative inputs. Deletion of either β1-integrin or ErbB4 resulted in defective alveolar development and Stat5 phosphorylation (Li et al., 2003; Long et al., 2003; Naylor et al., 2005), showing that they are enhancers of the pathway. Negative feedback regulation is provided by
members of the Socs family of proteins, which are induced by Prl signaling and negatively impact on Stat activation via different mechanisms (Oakes et al., 2008). Also caveolin 1 (Cav1), a structural component of caveolae, negatively regulates the Prl-Jak2-Stat5 axis (Park et al., 2002). One important downstream target of Prl (and P) signaling is RankL, which is a possible paracrine factor mediating proliferation in neighboring cells via induction of Cyclin D1. KO studies have shown that Cyclin D1 is indispensable for the massive proliferation during pregnancy (Sicinski et al., 1995). Another potential target of both Prl and P signaling is Wnt4, stimulating a variety of possible pathways and targets via β-catenin/TCF signaling. Recently, the Elf5 TF was identified as a target of Prl signaling and appears to play a major role in mediating alveolar morphogenesis, as expression of Elf5 can largely compensate for PrlR loss (Harris et al., 2006; Oakes et al., 2008). Finally, the gap junctions component connexin-26 is a direct target of Stat5 and necessary for junctional integrity of the forming alveoli (Ormandy et al., 2003).

Starting at mid-pregnancy differentiation of alveoli begins, which is also called secretory initiation or lactogenesis I. Morphologically, round and hollow alveoli form, consisting of a single epithelial layer surrounded by a discontinuous myoepithelial layer, thus ensuring direct contact to the basement membrane (Brisken and Rajaram, 2006; Richert et al., 2000). Intracellular lipid droplets are characteristic for this phase, as expression of milk proteins and lipids increases from then until parturition, while active secretion is inhibited by P. At this time, Prl functions are mainly taken over by placental lactogen, which was reported to bind to PrlR and GHR (Neville et al., 2002).

1.3.2 secretory activation and lactation

Lactogenesis II is induced around parturition by P withdrawal in the presence of high Prl levels, thus removing the inhibitory factor. In this phase tight junctions close completely to create the alveolar lumen, milk production is further increased, and milk components secreted into the lumen (Neville et al., 2002). Figure 4 shows a secreting alveolus with zoom on the milk production and secretion pathways within a luminal alveolar cell, processes that will be discussed below. Once the pups are born,
suckling stimulus and milk removal maintain lactation, with two hormones being involved: Prl acting on the luminal epithelial cells to maintain milk production and possibly acting as survival signal, and oxytocin, acting on the myoepithelial cells to induce contraction and milk ejection (Neville et al., 2002). Oxytocin is released upon a suckling stimulus and was shown to be absolutely required for milk release, as oxytocin-deficient mice fail to nurse their pups while milk is successfully produced in the alveoli. Importantly, injection of oxytocin restores successful release of milk, which can be used for experimental milk withdrawal (Young et al., 1996). It was further shown that oxytocin and milk removal are an indispensable stimulus for the proliferative burst that normally occurs early after parturition (Wagner et al., 1997).

1.3.3 milk: composition, production and secretion

Mouse milk is a very rich substance which consists of ~12% protein, 30% lipid and 5% lactose (Rudolph et al., 2007). This section will discuss how these components are efficiently and coordinately produced in the luminal alveolar cells and secreted into the lumen. Recent studies using microarray profiling revealed a
large number of genes regulated from pregnancy to lactation, involved in milk production and secretion (Rudolph et al., 2003; Rudolph et al., 2007).

Lactose is a disaccharide that is unique to milk and functions as the major osmole. It is composed of glucose and galactose and synthesized within the Golgi by lactose synthase (McManaman and Neville, 2003). The rate-limiting cofactor for this enzyme is α-lactalbumin (Soulier et al., 1997), a milk protein, which is one of the highly upregulated genes in lactation (Fig. 5). The increased demands of glucose can be met by upregulation of the glucose transporter GLUT-1 and downregulation of glycolysis enzymes, thus shuttling it towards lactose synthesis (and lipid synthesis, see below) (Anderson et al., 2007). Together with other soluble factors, like proteins, calcium, ions and water, lactose is secreted in Golgi vesicles via exocytosis into the lumen (Fig. 4).

Milk proteins are highly upregulated, starting already during pregnancy and increasing further after parturition, with the biggest changes in α-lactalbumin and δ-casein (Rudolph et al., 2003). TFs that regulate the expression of milk proteins also increase during pregnancy, such as C/EBPβ for β-casein and Ets for WAP (McKnight et al., 1995; Wyszomierski and Rosen, 2001). The caseins, together in a complex with calcium and phosphate, form micelles that are visible in electron microscopy (see Fig. 4; McManaman and Neville, 2003). Numerous studies have been performed, mostly using β-casein, to investigate mechanisms of milk protein regulation (reviewed in Rhoads and Grudzien-Nogalska, 2007). It is well established now that the increase in mRNA is due to increased transcription in combination with an increase in the stability of the mRNA. Furthermore, the strong increase in protein synthesis at lactation is not paralleled by increased mRNA levels, suggesting a translational control. Much evidence suggests that polyadenylation is an important mechanism in stabilizing mRNA and enhancing its translation (Rhoads and Grudzien-Nogalska, 2007).

Synthesis of milk lipids is probably the most remarkable task of the mouse mammary gland, as the mouse produces its whole body weight in fat during a full lactation (Rudolph et al., 2007). De novo triacylglycerol (TAG) synthesis takes place in the smooth endoplasmic reticulum (ERet) from fatty acids and a glycerol-
phosphate backbone (McManaman and Neville, 2003). Multiple enzymes are involved in this multistep process, many of which are highly upregulated during lactation (for metabolic map see Rudolph et al., 2007). Glucose again plays a prominent role and is processed by different pathways: first, it provides the TAG-backbone in a reaction involving Aldolase C; second, it generates energy (NADPH) via the pentose phosphate shunt and citric acid cycle; and third, it serves as a component in generation of fatty acid precursors (Rudolph et al., 2007). Once produced, the lipids bud from the smooth ERet, surrounded by one phospholipid layer of the ERet, and form cytoplasmic lipid droplets. Those can fuse while being transported through the cytoplasm, and finally get secreted in a unique budding process through the apical membrane, generating milk fat globules which are coated by an additional layer of plasma membrane and sometimes contain parts of the cytoplasm (see Fig. 4, left upper corner) (McManaman et al., 2006).

![Figure 5: Expression changes in the mammary gland during pregnancy and lactation, found by microarray or qPCR.](image)

**Figure 5**: Expression changes in the mammary gland during pregnancy and lactation, found by microarray or qPCR. Left panel: Milk proteins casein (Csn) α, β, γ, κ, milk fat globule-EGF-factor 8, whey acidic protein. Casein δ and α-lactalbumin on right axis. Right panel (normalized to P17): Regulatory factors SREBP-1 and Akt1, and glucose transporter Glut1. Average profile (dotted line, no scale) of 23 genes involved in fatty acid, TAG and cholesterol synthesis.

In the regulation of lipid synthesis, Akt1 (Fig. 5) is discussed to play a major role, as constitutive activation of Akt1 leads to excessive lipid synthesis (Schwertfeger et al., 2003), while in Akt1−/− mice the glucose transport and transcriptional regulation of
enzymes involved in lipid synthesis was impaired (Boxer et al., 2006). One major TF in milk lipid synthesis is SREBP-1c which regulates expression of various fatty acid biosynthetic enzymes (Goldstein et al., 2006). SREBP-1c is also highly upregulated during the transition to lactation and interestingly its active fragment is a member of the basic helix-loop-helix TF family, showing that many of the genes involved in lipid synthesis are regulated via sterol response elements as well as other TFs (Rudolph et al., 2007).

1.4 involution

Involution is the remodeling process following lactation that converts the gland back into a pre-pregnancy-like state. In experimental studies, synchronous involution can be induced by forced weaning, i.e. removal of the pups after about 10 days of lactation, which has been used to reveal many of the mechanisms and regulations during this phase (for detailed reviews see Green and Streuli, 2004; Watson, 2006). Forced involution can be separated in two distinct phases (Lund et al., 1996): the initial phase which is still reversible if pups are returned within 48 hours, and the second, irreversible phase that involves intensive tissue remodeling. Full regression and reorganization occurs after 21 days.

The first phase is characterized by accumulation of shed, caspase-3 positive cells in the lumen of the alveoli, which is obvious already after 12 hours (Watson, 2006). In experiments where a single teat was sealed it was found that mammary-intrinsic signals initiate involution, as only the sealed gland started to involute, and a major role seems to be accumulation of milk (Marti et al., 1997). This leads on one hand to accumulation of high concentrations of pro-apoptotic factors that normally get removed by nursing, and on the other hand to extensive stretching of the alveoli, which can possibly activate stretch receptors and disrupt cell-cell adhesions (Green and Streuli, 2004). Of the numerous signaling cascades involved in involution (gene expression analysis in Stein et al., 2007), a primary role was revealed for the LIF-Stat3 axis. Deletion of LIF, that is normally 30-fold induced within 12 hours of involution, leads to strongly delayed involution (Kritikou et al., 2003), similar to what occurs upon deletion of Stat3 (Chapman et al., 1999). Two important target
genes of Stat3 in the mammary gland are C/EBPδ and IGFBP-5, the latter inhibiting the IGF-1 mediated survival signaling (Green and Streuli, 2004). Interestingly, also c-Myc acts downstream of Stat3, and possibly mediates the apoptotic signaling (discussed in 3.1.2; Sutherland et al., 2006).

In the second and irreversible phase of involution the gland gets completely reorganized. The epithelial structures collapse, apoptotic cells get removed by phagocytosis (Monks et al., 2005) and stroma and adipocytes are regenerated. Important regulators during this phase are serine proteases and matrix metalloproteases (MMPs, for detailed review see Green and Lund, 2005). The MMPs, whose activity had been inhibited during the first phase by TIMPs, now remodel the ECM, thus leading to a second wave of apoptosis due to cell detachment and final collapse of the alveoli (Watson, 2006). One open question is how the post-lactational ductal structures are obtained, if ductal cells are refractory to apoptosis or if stem cells regenerate the ducts during involution (Green and Streuli, 2004).

1.5 stem cells

The presence of stem cells in the mammary gland was demonstrated around 50 years ago, in groundbreaking experiments by DeOme and colleagues (Deome et al., 1959). They developed an easy and elegant technique, which is still widely used today to test tissue for stem cell activity: the cleared fat pad transplantation (Fig. 6). In this technique, the gland of a 3 week old recipient mouse (immunocomprised or not) is ‘cleared’ by cutting out the endogenous epithelium, which at that point has not yet reached behind the lymph node. The remaining, epithelium-free fat pad serves as an environment for pieces of donor tissue. If the piece contained stem cells they will be able to repopulate the gland, forming a ductal tree, end buds and even alveoli if the recipient is mated.
Using the transplantation technique, numerous results pointed to the existence of mammary gland stem cells (reviewed in Smith and Medina, 2008): parts of the mammary gland were able to repopulate the fat pad with a normal tree, and differentiate under hormonal stimuli. This worked for several series of transplantations, although the cells showed signs of senescence after 5 to 8 rounds of transplantations. Furthermore, it was shown that every part of the mammary gland contained stem cell activity, independently from which stage of development (from virgin to lactating or involuted donor). By performing limiting dilution series and retrovirally marking of mammary epithelial cells it was found that at least two progenitors exist, generating either lobule-limited or duct-limited outgrowths, and that they derive from a single pluripotent precursor (Kordon and Smith, 1998; Smith, 1996). The current hypothesis from transplantation assays suggests that both the lobule- and duct-limited progenitors can give rise to luminal epithelial and myoepithelial cells, but the lobule progenitor cannot produce cap cells of TEBs while the duct progenitor cannot form cells contributing to alveolar development. Complementing this model are results from electron microscopy studies, that
identified morphologically distinct cells in the mammary gland (Chepko and Smith, 1997; Smith and Medina, 1988), with the ‘small light cell’ proposed to be a primary progenitor cell.

In the year 2006, two landmark studies changed the stem cell view and opened revolutionary new possibilities: the groups of Connie Eaves (Stingl et al., 2006) and Jane Visvader (Shackleton et al., 2006) identified new markers that can be used in FACS sorting to isolate potential mammary stem cells. Before that, FACS sorting was used to separate cells according to markers known from HSCs, like Sca-1 or their ability to efflux Hoechst dye (the so called side population, reviewed in Smalley and Clarke, 2005). By using CD24 (heat stable antigen) in combination with either CD29 (β1-integrin; Shackleton et al., 2006) or CD49f (α6-integrin; Stingl et al., 2006) populations highly enriched for mammary stem cell activity were isolated and analyzed in in vitro colony forming assays and in vivo transplantation assays. In both studies, very few numbers of those cells (down to one single cell with CD24^{+}CD29^{hi} or CD24^{med}CD49f^{hi}) were able to reconstitute fully developed outgrowths in serial transplants, thus fulfilling the ‘stem cell criteria’ of lineage differentiation and self-renewal. By further dissecting the CD24 expression levels and ER status in FACS sorted populations, the lab of Matt Smalley complemented the results of the other groups (Sleeman et al., 2006; Sleeman et al., 2007). Together they generated the current view of the mammary stem cell, which is thought to belong to the basal compartment (showing low expression of CD24), being negative for ER and high in expression of adhesion molecules CD29 and CD49f. The transplantation studies, FACS sorting, and use of transgenic mouse models finally led to different hypothesis of how the hierarchy of stem cells might look like in the mouse mammary gland, with one possible model shown in Figure 7.
Figure 7: Stem cell hierarchy in the mouse mammary gland. One possible model based on numerous experimental findings.

Note that various different models exist for the stem cell hierarchy, and it has yet to be determined which one comes closest to the situation in the mouse. Another model suggests the existence of a common ‘luminal progenitor’ that gives rise to both ductal and alveolar cells (Visvader and Lindeman, 2006). It is also not yet fully understood how to combine these models with the experimental findings discussed above, that suggested the existence of a duct-limited progenitor and a lobule-limited progenitor, which could both give rise to luminal epithelial and myoepithelial cells, either in ducts or in lobules. The question about the stem cell niche also remains still to be clarified, together with the identity and location of the stem cell. The possible signals and interactions that might be involved between mammary stem cells and their niche have recently been discussed, as compared to systems that are well understood like the hematopoietic system and the skin (Tanos and Brisken, 2008).
Interesting results were obtained from studies with Rosa26-lox-STOP-lox-LacZ reporter mice that can be used to permanently label and track cells in vivo. When crossed with mice carrying a Cre transgene, recombination of the floxed Stop codon will occur in Cre expressing cells, and the cells will from that moment on continuously express lacZ (β-galactosidase) and can be labeled in blue by X-Gal staining. Using a Cre recombinase under a milk protein promoter (WAPCre), which is only expressed starting mid-pregnancy in differentiating luminal epithelial cells, a population of (blue) WAP expressing cells could be identified that did not undergo apoptosis during involution and could still be stained in a parous, non pregnant female (Wagner et al., 2002). This population was termed Pi-MECs (for parity-induced MECs), and the cells were thought to arise during pregnancy and eventually even from de-differentiation of a fully committed, secretory cell (Fig. 8, left panel). They were shown to serve as progenitors in subsequent pregnancies, contributing to both luminal and myoepithelial cells in a second pregnancy (Fig. 8, right panel). In transplantation studies it was found that Pi-MECs are self-renewing and form lobule-limited outgrowths (Booth et al., 2007; Wagner et al., 2002), suggesting they are the lobule-limited progenitors. In addition, labeling them with GFP instead of LacZ expression, which enabled FACS analysis, showed that the Pi-MECs reside within the CD49f<sup>hi</sup> population of the mammary gland (Matulka et al., 2007). Most important, they were finally found to exist in the virgin gland and were shown to be responsive to hormonal stimulation (Booth et al., 2007), supporting the hypothesis that they could be identical to the ‘lobule-limited progenitors’ proposed by Smith and colleagues or to the ‘alveolar progenitor’ or ‘luminal progenitor’ proposed by Rosen, Visvader and others. The identification of Pi-MECs in virgin glands led to their renaming into ‘parity-identified’ MECs, as they were not generated by pregnancy but just labeled by WAPCre expression during pregnancy. Nevertheless it is important to be aware of the fact that those cells are partially differentiated (WAP expression) yet still pluripotent and surviving involution.
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To give a short outlook on the situation in human breast: studies have been intensified during the past few years and FACS analysis and new markers have been used for human cells (for a recent review on markers used for mouse and human mammary epithelial cells see Stingl, 2009). In comparison to the mouse, other markers are used for the isolation of potential human mammary stem cells: EpCAM (epithelial cell adhesion molecule), MUC1 (a luminal cell-specific glycogen), ALDH1 (aldehyde dehydrogenase 1) and also CD49f. Testing cell populations in transplantation assays suggested that mammary stem cells show a EpCAM<sub>low</sub>CD49f<sub>hi</sub>MUC1<sup>-</sup>ALDH1<sup>+</sup> phenotype, implying that their position is basal, as also suggested for mouse. A proposed hierarchy for mouse and human mammary stem cells based on surface markers can be found in (Stingl, 2009).
1.6 breast cancer

One reason to study mammary gland is not only to gain insight into the pathways regulating its function and development, but also to understand the mechanisms leading to breast cancer and ultimately to find new treatments for this disease. This section gives a short introduction to breast cancer, concentrating on the different subtypes and the available treatments.

1.6.1 incidence of breast cancer and molecular subtypes

Breast cancer is the most common cancer in women worldwide and the most important cause of neoplastic deaths among women (World Cancer Report (WCR) 2008). The incidence is especially high in North America but also in Northern and Western Europe (Fig. 9), with high rates of 80-90 per 100,000. Alone in Switzerland, over 5,000 women are diagnosed yearly (average from 2001 to 2005) with breast cancer, which comprises almost 15% of all new cancers and 32% of all cancers in women per year. In addition, breast cancer shows the highest cancer mortality (1,300 per year), with 1 out of 5 cases (in women) of cancer deaths being caused by breast cancer (Source: Krebsliga Schweiz, 2008). Some good news is, that although the incidence for breast cancer is increasing, mortality is slowly decreasing, which is mainly due to better screening and treatment options (WCR 2008).

*Figure 9: Worldwide incidence of breast cancer.* The map refers to women of all ages, incidence is given as numbers per 100,000. Note the high incidence in Western Europe.
Breast cancer is a very heterogeneous disease, which in the past was mainly diagnosed and described using multiple histological features (such as immunohistochemistry (IHC) status of certain markers, proliferation etc.) and light microscopy. Over 80% of neoplasms in the breast arise from ductal rather than lobular epithelium (WCR 2008) and these roughly are described as atypical ductal hyperplasia, intraductal carcinoma (or ductal carcinoma in situ, DCIS) and invasive ductal carcinoma (histological classification can be found in a Review from Andrew Fischer on the ‘Biology of the Mammary Gland’ webpage, see http://mammary.nih.gov/reviews/tumorigenesis/Fischer001/index.html).

Furthermore, tumors were characterized by expression of steroid and GF receptors, such as ERα or ErbB2, by their lymph node status, and histological grade (differentiation status). At the beginning of the 21st century, new results from microarray analysis allowed the identification of robust subtypes that were defined by gene expression, termed ‘molecular portraits’ of breast cancer (Perou et al., 2000; Sorlie et al., 2001). Interestingly, those subtypes are further characterized by other clinical markers, such as ERα or ErbB2 expression, which led to the following nomenclature (Fig. 10A): ‘basal-like’ (showing highest expression of basal keratins 5, 6, 17 and/or laminin), ‘ErbB2+’ (overexpression of ErbB2), ‘normal breast-like’ (a gene expression similar to normal breast samples) and ‘luminal’ (expressing luminal keratins 8/18 and being positive for ERα). The luminal, ER+ subtype can be further divided into luminal A (showing the highest ER expression), and luminal B and C (often combined, showing low to mediate ER expression, luminal C showing some similarity to basal-like and ErbB2 subtypes). Importantly, when applying these subtype division to a group of 49 breast cancer patients with uniform treatment, a clear correlation with clinical outcome was found (Fig. 10B), in which the luminal A subtype showed the best, and the basal subtype the worst overall survival (Sorlie et al., 2001). The basal subtype is sometimes also called ‘triple negative’, as it is negative for expression of ErbB2, ER and PR. Interestingly, tumors with BRCA1 mutations predominantly fall into this subtype, and are associated with poor prognosis (Sorlie et al., 2003). When including results from more recent studies, it emerged that 5 subtypes are repeatedly observed, excluding the luminal C (Hu et al.,
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2006). More about c-Myc and the different subtypes of breast cancer can be found in chapter V.

1.6.2 strategies for treatment of breast cancer

Before the detailed analysis of breast cancers by gene expression, mostly IHC (or in situ hybridization, FISH) was used to check for expression of certain markers that would allow selection of a specific treatment as opposed to normal chemotherapy. As such, ER and ErbB2 were the first two markers/molecules against which specific therapy for a pre-selected group of patients was available.

The ‘oldest’ available targeted therapy for ERα positive breast tumors is the anti-estrogen or SERM (selective estrogen-receptor modulator) tamoxifen, which was synthesized in 1966 in Great Britain, and approved for treatment of metastatic breast cancer in 1973 (Great Britain) and 1977 (United States) (Jaiyesimi et al., 1995). Its mechanism of action was unsolved for a long time, but it is now clear that tamoxifen acts as an estrogen antagonist (recruiting corepressors to the ER) in the breast, while it acts as an agonist of estrogen signaling (recruiting coactivators) in other tissues like the endometrium (Riggs and Hartmann, 2003). While tamoxifen was the ‘gold standard’ of treatment of ER+ tumors for many years, resistance against endocrine therapy usually develops. The mechanisms contributing to resistance include a ‘positive feedback’ crosstalk between ER and GF receptor which enhances the
agonistic effects of tamoxifen and thus tumor growth (numerous reviews available about endocrine resistance, see e.g. Clarke et al., 2003; Osborne et al., 2005). An alternative treatment strategy is provided by aromatase inhibitors such as letrozole, which block ER signaling by preventing estrogen synthesis (Carpenter and Miller, 2005). Together with tamoxifen, they form the base for current treatment in the metastatic as well as the adjuvant setting (Harichand-Herdt et al., 2009).

Amplification of ERBB2 is found in approximately 20-25% of primary breast tumors and was the first consistent alteration correlated with breast cancer (Berger et al., 1988; Slamon et al., 1987). To date, two different successful treatments are available to patients with ErbB2-overexpressing breast cancer: antibodies, targeting the extracellular domain of the receptor and TKIs (tyrosine kinase inhibitors) that inhibit the intracellular kinase activity (recently reviewed in Hynes and MacDonald, 2009). The monoclonal antibody trastuzumab (Herceptin) was approved in 1998 for treatment of metastatic ErbB2+ breast cancer and has since then developed to an important therapeutic option also in the adjuvant setting (for a detailed review of trastuzumab history see Ross et al., 2009). The second class of inhibitors includes lapatinib, which is a dual EGFR/ErbB2 TKI. One big problem that remains is the resistance that can develop in response to therapy, which in the case of ErbB2 overexpressing breast tumors often seems to involve activation of PI3K/Akt signaling pathways (Hynes and MacDonald, 2009). Thus, there is a constant need for new targets, new drugs and new combinations of treatments. A variety of promising compounds targeting ErbB2 is in Phase I and II clinical trials; alternative strategies to enhance anti-ErbB2 therapies have already made it into Phase II and III, like targeting Src or VEGF, respectively (Bedard et al., 2009).

The use of gene expression analysis to classify and characterize breast tumors has opened the door for new and tailored therapies. By identifying activated pathways and molecules, specific inhibitors (or combinations of inhibitors) can be directed against specifically upregulated or activated targets. This is of special interest in the case of the basal breast cancer subtype as no targeted inhibitor like tamoxifen or trastuzumab can be used for treatment. Very recently, a study used gene expression analysis to identify pathway activation among different subtypes and to predict
sensitivity to chemotherapeutic agents (Bild et al., 2009). The results demonstrate that a large fraction of the basal subtype exhibits EGFR pathway activation, with an inverse pattern of Ras or Src pathway activation. Similarly, there was a big heterogeneity within this subtype in gene expression in response to chemotherapy, identifying clear clusters that would be sensitive to one agent but resistant to another. There is clearly still a big demand for new drugs, but the recent advances in technologies are a reason to hope that understanding and treating breast cancer will be even more successful in the future.
II. The multiple roles of c-Myc

The proto-oncogene c-MYC is the most extensively studied member of the myc family of oncogenes (which consists furthermore of N-Myc and L-Myc). c-MYC was first discovered as the cellular homologue of the transforming sequence of an avian virus, which was named myc, for myelocytomatosis (reviewed in Varmus, 1984). Interest and research investigations in Myc grew tremendously when it was subsequently found to be overexpressed in Burkitt’s lymphoma and various other human malignancies (for a recent timeline on Myc in cancer research see Meyer and Penn, 2008). This section will try to give an overview on Myc in general, its regulation, its connection in signaling networks, and its biological functions. For a more detailed description, the reader is kindly referred to PubMed, where to date more than 20,000 articles can be found on Myc.

2.1 structure and regulation

Myc expression is regulated by many signals on multiple levels, from transcriptional/post-transcriptional to translational/post-translational, with a very short half life for mRNA and protein (20-30 min). The levels of c-Myc mRNA and protein are usually low in normal cells so that already small changes can exert a big effect. This part will focus on the tight and fast regulation of Myc transcription, as well as protein modifications influencing its activity or stability.

The human c-MYC gene which is located on 8q24 harbors four different promoters (P0 to P3, of which P2 is the preferential one for 75 - 90% of transcripts) (Wierstra and Alves, 2008). The c-Myc promoters are so complex with containing binding sites for such a large number of TFs, GFs or other factors that a recent review used 220 pages to describe the current knowledge about the Myc promoters (Wierstra and Alves, 2008). To mention only a few, the c-Myc promoter region contains TF binding sites for Myc (autosuppression), β-catenin/TCF, Notch/Cbf1, TGFβ/Smads, Fos/Jun, E2F, Stat3 and NF-κB. Furthermore, and especially interesting in the mammary gland, the c-Myc promoter region was shown to contain an estrogen as
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well as a progesterone responsive element (ERE, Carroll et al., 2006; and PRE, Moore et al., 1997). Finally, various signaling pathways influence c-Myc expression, leading to a sharp increase upon proliferation stimuli, which can be GFs, mitogens, cytokines, hormones, vitamins and other ligands.

Before discussing post-translational modifications of the c-Myc protein, its structure shall be discussed. Translation of the c-Myc protein can start from two alternative translational start sites (Wierstra and Alves, 2008). This generates the two major proteins c-Myc1 (p67, starting from the CTG codon) and c-Myc2 (p64, starting from the AUG codon), with the latter being the predominant form in growing cells. The full-length c-Myc protein contains 2 domains: the N-terminal domain (NTD) and the C-terminal domain (CTD, Fig. 11). The NTD is also called transactivation domain because it is the major regulatory region necessary for all transactivating and transrepressing activities. It contains three conserved motifs known as Myc boxes (MBI - III), of which MBII was shown to be the highest conserved motif and the most essential for c-Myc’s biological functions and for cofactor binding (Cole and Cowling, 2008; Ponzielli et al., 2005).

![Figure 11: Protein structure of c-Myc.](image)

The CTD, also termed DNA-binding domain, contains another Myc box (MBIV), the primary nuclear localization signal (NLS) and the essential basic motif (B) for binding to the E-box DNA sequence (CACGTG). In addition, it contains the helix-loop-helix leucine zipper (HLH-LZ) domain, which puts Myc into the superfamily of B-HLH-LZ TFs, and which is required for heterodimerization with its binding partner Max (Max will be discussed in the next chapter). It was shown that via ‘leaky scanning’ mechanism translation may start from alternative downstream AUG
codons and produce different truncated (‘small’) forms termed c-MycS (Spotts et al., 1997). As this form is N-terminally truncated (lacking MBI), it can still heterodimerize with Max, but has no transactivating function.

The Myc protein is regulated by various post-translational modifications including phosphorylation, acetylation and ubiquitinylation (reviewed in Vervoorts et al., 2006). Two important phosphorylation sites lie within MBI, Thr-58 and Ser-62, which are regulated by PI3K and Ras signaling, respectively. Phosphorylation of Ser-62 upon activated Ras/MAPK signaling leads to stabilization of c-Myc and eventually to effects on Myc-dependent gene expression. In contrast, phosphorylation of Thr-58 by GSK3 induces a series of events resulting in Myc degradation. Importantly, GSK3 is inhibited by PI3K/Akt signaling, and phosphorylation of Thr-58 needs phospho-Ser-62 as a prerequisite, thus providing a tight regulation of Myc function and half life. Moreover, c-Myc is subject to control by different ubiquitin-ligase complexes, which are dependent or independent of its phosphorylation status. Different ubiquitinylations can have transcription activating effects before actually leading to degradation of the Myc protein. Finally, c-Myc is a substrate for several histone acetyltransferases that it recruits leading to acetylation at several different lysines. The function of acetylation is not yet fully understood, but it is thought to be stabilizing, as it might interfere with ubiquitination of lysines.

2.2 the Myc/Max/Mad transcriptional network

c-Myc is only one of a large number of B-HLH TFs that form different dimers and act as positive or negative regulators of polymerase (Pol) II mediated transcription, depending on the cellular context. The central player of the network is Max (Myc associated protein X), as it can form either homodimers or heterodimers, depending on availability of dimerization partners, with Myc but also with Mnt, the Mxd proteins (formerly called Mad proteins) and Mga (Grandori et al., 2000; Hooker and Hurlin, 2006). All complexes bind to E-boxes (see above), but by recruitment of different cofactors they exert different effects on transcription (with Max-Max homodimers having no transcriptional activity). The network is tightly regulated (and well studied) during cell cycle progression, where the levels of its components
change, and gets deregulated in a tumor setting if one of the components is strongly upregulated or lost. A simplified scheme of the network is shown in Figure 12.

As mentioned above, Max is the central player of the network. Under physiological conditions, Max protein shows a very slow turnover, and it seems to be expressed at rather constant levels during the cell cycle. The same is true for Mnt, which led to a model where Mnt-Max complexes might form a ‘ground state’ and repress target gene expression (Hooker and Hurlin, 2006; Hurlin and Huang, 2006). c-Myc, which is highly upregulated in response to mitogens at the entry into the cell cycle, replaces Mnt and forms complexes with Max, thereby activating transcription of multiple targets (discussed below). Although Mnt-Max and Myc-Max complexes have been shown to coexist in a variety of proliferating cell types, Max is not very abundant and might be limiting in certain settings, especially when dimerization partners are expressed at very high levels. Interestingly when looking at tumorigenesis, particularly loss of Mnt can result in phenotypes that resemble Myc overexpression (e.g. in mammary glands), showing indeed their antagonistic competition for Max binding (Hurlin et al., 2003; Nilsson et al., 2004; Toyo-oka et al., 2006). As cell cycle progresses, c-Myc levels go down, and levels of Mxd (Mad) proteins, which correlate with terminal differentiation, rise and they form complexes
with Max, again repressing target gene transcription (Grandori et al., 2000; Hooker and Hurlin, 2006). The transcriptional activities involving c-Myc will now be discussed in more detail.

### 2.2.1 activation and repression of polymerase II targets

Myc-Max heterodimers bind to canonical (CACGTG) but also non-canonical E-boxes. A number of studies have been devoted to identifying and confirming c-Myc-binding sites and transcriptional targets. To summarize, these studies suggest that c-Myc binds 15% of all genes (around 25,000 binding sites in the genome!) (for reviews see Adhikary and Eilers, 2005; Eilers and Eisenman, 2008; Patel et al., 2004). One interesting finding was that c-Myc binding takes place almost exclusively in a certain chromatin context termed euchromatic islands which contain active histone modification marks that are characteristic for actively transcribed or previously transcribed loci (Guccione et al., 2006). This widespread DNA binding can be controlled and modulated by low protein levels and rapid turnover of c-Myc, which implies that c-Myc binding at certain sites is transient and also the exchange of cofactors can take place rapidly (discussed in Eilers and Eisenman, 2008). The cofactors recruited by c-Myc for transcriptional activation are also numerous and include deubiquitinating and demethylating enzymes, histone acetyltransferases and other histone modifying proteins (Eilers and Eisenman, 2008). One cofactor that was shown to be essential for c-Myc mediated transformation is the adaptor protein TRRAP (TRansactivation/tRansformation Associated Protein; Cowling and Cole, 2006).

Moving on to the transcriptional targets of c-Myc, it is a fact that c-Myc acts as a relatively weak activator with an average expression change of 2-fold of its target genes. Nevertheless c-Myc affects multiple cellular functions due to the large number of targets. A list of direct genomic targets, mostly derived from studies in Burkitt’s lymphoma, rat fibroblasts and Drosophila, can be found at http://www.myc-cancer-gene.org/index.asp (Zeller et al., 2003), which to date contains almost 1700 targets. When distributing the targets according to functional categories it becomes clear that c-Myc affects specific classes of genes from cell cycle regulation, metabolism,
protein biosynthesis and cell adhesion/cytoskeleton (Dang et al., 2006). More recently, also the miRNA cluster miRNA-17-92 was shown to be upregulated by c-Myc (O'Donnell et al., 2005). Deregulation of c-Myc furthermore affects apoptosis, differentiation, angiogenesis and genomic instability (Meyer and Penn, 2008; Ponzielli et al., 2005).

As mentioned above (Fig. 10), transcription is also negatively regulated by the Myc/Max/Mad network. Mnt, Mxd and Mga in complex with Max compete with Myc-Max for binding to the same E-boxes. Repression of transcription occurs via recruitment of the SIN3 adaptor protein and histone deacetylases (Adhikary and Eilers, 2005). Myc-Max complexes can also repress transcription; indeed, 10 - 25% of the identified c-Myc targets are repressed rather than activated (Zeller et al., 2003). Repression does not occur via DNA binding, but rather via interaction with, and inhibition of, other transcriptional regulators, like Sp1, Smad2/Smad3 and Miz-1 (Myc-interacting zinc finger protein 1; Eilers and Eisenman, 2008). The well studied Cdk-inhibitors p15INK4b (Seoane et al., 2001) and p21CIP1 (Wu et al., 2003) were shown to be repressed by c-Myc via interaction with Miz-1 on core promoters. This binding has two effects: passive interference with the interaction between Miz-1 and coactivators and active recruitment of corepressors (such as DNA methyltransferase) to Miz1 (Brenner et al., 2005). Another recent study showed that in contrast to the miRNA-17-92 cluster, c-Myc represses a large number of other miRNAs, including members of the let7 family (Chang et al., 2008). A summary of Myc’s transcriptional activity is shown in Figure 13.
2.2.2 activation of transcription by Pol I & III

In addition to the large number of Pol II transcribed targets, another interesting aspect is that c-Myc also regulates transcription by Pol I and Pol III (Fig. 13), thus linking cell growth and metabolism to cell division (reviewed in Oskarsson and Trumpp, 2005). Pol I transcribes the rRNA gene to produce a 45S eukaryotic pre-rRNA precursor, which gets further processed into the 18S, 5.8S and 28S rRNA. c-Myc was shown to directly activate rRNA transcription via binding of several E-boxes in the promoter region and recruitment of TRRAP (Arabi et al., 2005; Grandori et al., 2005), similarly to what was shown for activation of Pol II driven transcription. In contrast, activation of Pol III transcription occurs via a different mechanism. The Pol III transcribed genes for tRNA and 5S rRNA do not contain a DNA sequence that
is directly recognized by c-Myc. It was shown that c-Myc activates Pol III transcription by interaction with the Pol III specific factor TFIIIB (Gomez-Roman et al., 2003), involving recruitment of TRRAP and histone acetyltransferases (Kenneth et al., 2007). Together with Pol II transcribed targets like ribosomal proteins, translation factors and rRNA processing factors, this makes c-Myc a master regulator of protein synthesis (White, 2005).

2.3 other functions of c-Myc

Recently, other functions of c-Myc have been described that do not result in transcriptional changes of single targets but affect other cellular aspects. In addition to changing histone modifications to activate target gene transcription, it was shown that c-Myc (and also N-Myc) acts as a global regulator of chromatin (Knoepfler et al., 2006). In this study, loss of Myc resulted in a decrease in active and an increase in repressive chromatin marks and expanded heterochromatic regions. This was mechanistically explained by lower levels of the histone acetyltransferase GCN5, a likely Myc target gene. Another function of c-Myc with a very broad effect is the ability to regulate DNA replication. It was shown that independent of its transcriptional activity, c-Myc binds to the pre-replicative complex and increases activity at DNA replication origins (Dominguez-Sola et al., 2007). The authors further speculate that this is not only another mechanism for c-Myc to drive proliferation but also a way how deregulated c-Myc may promote DNA damage and genomic instability by inducing DNA replication stress. Finally, c-Myc was found to influence translation of target genes and non-target genes via two different mechanisms (reviewed in Cole and Cowling, 2008). First, c-Myc induced global (target gene independent) phosphorylation of the C-terminal domain of Pol II, which is necessary to release the paused Pol II and drive elongation of transcription (Cowling and Cole, 2007). This study further showed that c-Myc increased mRNA 5’-cap methylation of target but also non-target genes, which led to increased polysomal loading of those mRNAs and increased translation rate. Interestingly, the same group showed this year that cap methylation occurred very frequently for c-Myc targets while being absent from control genes like GAPDH, and that the
increase in cap methylation (from 1.5- to 8.9-fold) was usually higher than transcriptional upregulation (from 1.4- to 2.2-fold) (Cole and Cowling, 2009). This finding led to the new hypothesis that mRNA cap methylation might even be the major mechanism underlying c-Myc regulation of gene expression.

2.4 c-Myc in human cancer

Studies on c-Myc in human malignancies started in the early 1980s, when c-MYC was found to be the human cellular homologue of the transforming sequence of the avian myelocytomatosis retrovirus MC29 (recently reviewed in Meyer and Penn, 2008). It was an interesting finding that in contrast to other oncogenes like Ras, c-Myc seemed not to be generally activated via mutations in the coding sequence, but rather by other mechanisms. Two mechanisms identified for c-Myc were insertional mutagenesis, where c-Myc was activated by viral promoter insertion (Hayward et al., 1981; Payne et al., 1982), and translocation, where the c-MYC locus is translocated to Ig heavy and light chain genes, the dominating deregulation found in Burkitt’s lymphoma (Dalla-Favera et al., 1982). In solid tumors, aberrant levels of c-Myc are obtained via amplification or overexpression and occur with high frequency in many tumor types (a table of Myc deregulations in human tumors can be found in Vita and Henriksson, 2006). As c-Myc itself is a target of numerous signaling pathways, TFs, hormones etc. (see above), its overexpression can occur on various different levels and thus is a likely event in any human cancer: c-MYC mRNA expression gets enhanced via activated GFs, receptors or hormones (e.g. EGF, Wnt, E), via activated second messengers (e.g. Src, Ras, Akt) and via activated TFs (e.g. Notch, β-catenin/TCF) (Meyer and Penn, 2008). Furthermore, factors influencing Myc mRNA and protein stability and turnover can be deregulated in cancer and thus change Myc protein levels. Some examples of mechanisms that occur in breast cancer are discussed in Chapter V.
III. Studying c-Myc function in mouse models

This section will describe various in vivo studies that have helped to clarify many different functions of c-Myc not only during embryogenesis, organ development and homeostasis, but also in tumorigenesis. For the mammary gland, different transgenic overexpression models exist, which are described in the first part of this section; however, there is not a lot known about the effects of c-Myc loss in this organ. In contrast, in many other organs like bone marrow, intestines and skin, conditional KO (CKO) of c-Myc has been performed, and this will be discussed in the second part of the section. Finally, c-Myc has also been tested as a tumor target in mice, via genetic downregulation or chemical treatment, which is summarized in the last part of the section.

3.1 c-Myc overexpression in the mammary gland using transgenic mouse models

3.1.1 Tumorigenic phenotypes

As c-Myc is known to play a role in development and progression of many human cancers, including breast cancer, it has been widely studied in different transgenic mouse models (reviewed in Jamerson et al., 2004). The first transgenic model generated was c-Myc expression under the mouse mammary tumor virus (MMTV) promoter (Stewart et al., 1984). This regulatory element shows activity in mammary epithelium of virgin mice and is greatly enhanced by hormones during pregnancy (Donehower et al., 1981). In this study, the incidence of mammary adenocarcinomas was 100% for multiparous and around 50% for virgin animals. The fact that the tumors arose spontaneously and not uniformly in all mammary glands suggested that c-Myc overexpression alone was not sufficient for transformation in vivo (Stewart et al., 1984). Another transgenic model utilized the WAP promoter, where transgene expression is low during each estrus cycle and high in secretory alveolar cells during late pregnancy and lactation (Sandgren et al., 1995; Schoenenberger et al., 1988). As expected from the expression pattern of the WAP promoter, both studies reported that
pregnancy/lactation was indispensable for tumor formation (80 – 100% incidence in multiparous females) and virgin animals stayed tumor free during 4 months. Interestingly, the tumors showed constitutive milk protein expression of WAP-Myc as well as endogenous WAP and β-casein, independently of lactation (Schoenenberger et al., 1988). Recently, new insights were gained from an inducible tetracycline-dependent model, where c-Myc expression in mammary and salivary gland can be induced at any time by giving doxycycline to the mice (MMTV-rtTA/TetO-Myc; Boxer et al., 2004; D'Cruz et al., 2001). Induction of transgene expression led to tumor formation with high incidence (86% after 22 weeks) as well as rapid regression in around 50% of tumors within 2 weeks after doxycycline removal. Interestingly, many of the non-regressing tumors as well as some of the relapsing tumors displayed activating mutations in Kras2 or Nras. Thus, mutant Ras seems to facilitate an escape from Myc dependence in Myc-induced breast tumors, which is in contrast to most other Myc-induced tumors, that fully regress upon Myc inactivation (reviewed in Arvanitis and Felsher, 2006). The synergy between MMTV/WAP-c-Myc and other oncogenes was also examined in various bitransgenic models (reviewed in Jamerson et al., 2004), using for example activated Ras or Neu and thereby greatly accelerating tumor formation.

### 3.1.2 non-tumorigenic phenotypes

The above mentioned doxycycline inducible model (D'Cruz et al., 2001) was further used to investigate non-tumorigenic effects of aberrant c-Myc expression at different times in mammary gland development. Expression of c-Myc was shown to be tightly regulated in the mammary gland, being high during early pregnancy, almost undetectable during lactation and upregulated again in involution (Master et al., 2002; Strange et al., 1992). Two studies used the inducible transgenic model to describe effects of c-Myc overexpression during pregnancy (Blakely et al., 2005) and involution (Sutherland et al., 2006). To date, these are the only non-tumorigenic Myc studies in the adult mammary gland, thus they will be discussed in more detail.

CKO studies have shown how the effects of c-Myc depend to large extent on the time when the deletion occurred. Microarray data revealed that c-Myc expression in
the mammary gland rises nearly 3-fold from virgin until day 6 of pregnancy, reaches basal levels again by day 18 of pregnancy, and is 3- to 4-fold downregulated during lactation. The Chodosh lab found that a very short c-Myc induction from day 12.5 to day 15.5 of pregnancy is necessary and sufficient to result in complete pup death within 24h after birth. The lactation failure was found to result from precocious lactogenic development leading to apoptosis even before parturition. As a primary effect only 24h after c-Myc induction, the proliferation rate of mammary epithelial cells was induced 10-fold, followed by premature activation of Stat5 and high expression of milk proteins. Mechanistically, this was explained by a rapid downregulation of Cav1, which is a target of transcriptional repression by c-Myc (Park et al., 2001) and a negative regulator of the Prl-Jak2-Stat5 pathway (Park et al., 2002). Interestingly, aberrant c-Myc expression also induced high epithelial apoptosis within 24h after doxycycline treatment and a second wave of apoptosis later in pregnancy. This second phase was caused by milk stasis and directly led to precocious involution via activation of Stat3 and Tgfβ3 pathways. This study brought some insight into the effects of specific and restricted overexpression of c-Myc in the mammary gland, including some interesting potential targets and the finding that overall proliferation and differentiation were accelerated by abnormal c-Myc levels.

The second study, published by the Visvader lab, utilized the inducible c-Myc model to address the role of c-Myc in involution. They initially investigated conditional Socs3 KO mice (WAPiCre;Socs3−/fl) which undergo precocious involution. The mammary glands of these mice display increased Stat3 activation during lactation, where it is normally repressed by Socs3, followed by significantly elevated levels of the pro-apoptotic regulators Bax and Bak. Interestingly, also c-Myc, which is a Stat3 target, and the pro-apoptotic c-Myc target genes E2F-1 and p53 were found to be upregulated in Socs3-deficient glands. To test whether c-Myc is the main mediator of the involution phenotype observed in Socs3 CKO mice, they induced c-Myc expression in the transgenic model at day 8 of lactation followed by forced involution 2 days later (and continuing doxycycline treatment for 2 more days). Following 4 days of c-Myc induction, involution was found to be dramatically accelerated, including increased apoptosis and elevated levels of Bax, p53 and E2F-1.
These results show that c-Myc has a clear role in involution, at least from overexpression studies.

3.2 c-Myc downregulation in various systems

3.2.1 full knockout: embryonic lethality and partial rescue

Starting in the 1990s, an increasing number of reports investigated not only tumorigenic functions of c-Myc, but focused on its physiological roles in embryogenesis and development. The first attempt to create homozygous KO mice revealed that a c-myc null mutation is embryonic lethal between 9.5 and 10.5 days of gestation (Davis et al., 1993). Homozygous embryos analyzed before that showed a strong general delay in development as well as a reduction in size and other abnormalities. Interestingly, also adult females heterozygous for the null allele revealed a phenotype, namely reduced fertility. However, the primary defect caused by c-myc loss was not determined in this report. In 2001, a series of mice with stepwise decreasing c-Myc levels was published, including a new null allele and a floxed allele (Trumpp et al., 2001). This study confirmed the embryonic lethality of complete c-Myc loss and showed further that incremental reduction of c-Myc levels lead to incremental decrease in body mass. Mice heterozygous for the c-myc null allele never reached the size of normal littermates with all organs being reduced in size as well. Using flow cytometry it was found that not cell size but cell number was reduced in most organs, in contrast to Drosophila dmyc mutants, which are smaller due to hypotrophy (Johnston et al., 1999). The discussion whether c-Myc affects cell number and/or cell size will appear also in other c-Myc CKO studies. The c-myc<sup>fl/fl</sup> mice generated in 2001 (de Alboran et al., 2001; Trumpp et al., 2001) were intensely studied using transgenic mice carrying Cre recombinase under various promoters for organ specific Myc deletion (for examples, see below). Recently, by using a Sox2-Cre recombinase, which leads to c-Myc loss specifically in the epiblast, it was shown that the observed embryonic lethality was due to the indispensable requirement of c-Myc in the placenta and the hematopoietic system (Dubois et al., 2008; He et al., 2008). Most other non-hematopoietic tissues were able to proliferate and develop even in the absence of c-Myc, until the embryos died before day 12 of gestation due
to severe anemia. The specifically important role of c-Myc in the hematopoietic system was investigated in great detail, which will be discussed in the next section.

### 3.2.2 conditional knockout in the hematopoietic system

As mentioned above, c-Myc plays a pivotal role in the fetal hematopoietic development. Although definitive hematopoietic stem cells (HSCs) were generated, they were non-functional and unable to expand in vitro or in vivo in mutant liver (Dubois et al., 2008). While expression of adhesion molecules was not altered and migration to the liver seemed not affected, p21<sup>CIP1</sup> was found to be upregulated in mutant stem/progenitor cells. A different mechanism for c-Myc was found in adult HSCs (Baena et al., 2007; Wilson et al., 2004). Deleting c-myc perinatally in liver, spleen and bone marrow (using the IFNα-inducible Mx-Cre transgene) leads to severe anemia and loss of committed hematopoietic lineages, while HSCs are accumulating in the bone marrow. While Baena et al. reported overexpression of both p21<sup>CIP1</sup> and N-Myc in one subset of HSCs, Wilson et al. found a striking upregulation of adhesion molecules in another subset. They proposed a model where loss of c-Myc in HSCs does not impair their self-renewal and proliferative capacity, but due to high expression of adhesion molecules the cells cannot leave the stem cell niche and fail to differentiate. Supporting this model was also the finding that c-Myc-deficient HSCs were fully able to differentiate ex vivo, albeit with only minimal expansion in the transient amplifying cells. Recently, it was shown that N-Myc plays a major role in c-Myc-deficient bone marrow (Laurenti et al., 2008). While loss of N-Myc alone did not affect HSCs, simultaneous deletion of c- and N-Myc led to a rapid depletion of the HSC pool due to aberrant proliferation and increased apoptosis. This is one example of how N-Myc can at least in part functionally replace c-Myc in vivo.

### 3.2.3 conditional knockout in the intestines

The intestinal epithelium is a well organized, highly regenerating epithelium, where stem cell proliferation and differentiation are coupled to migration within the intestinal units called crypts (Sancho et al., 2003). As c-Myc is a well established target of the Wnt/β-catenin pathway in intestinal crypts (van de Wetering et al.,
Introduction: c-Myc in mouse models

(2002), two groups investigated the effects of c-Myc loss in this organ using different Cre transgenic mice. Deletion of c-Myc in the small intestine using the Villin promoter in 1 week old mice led to a surprisingly mild phenotype, with an early transient decrease in crypt number but a complete absence of long-term defects in adult intestines (Bettess et al., 2005). In contrast, deletion of c-myc using the Cyp1a promoter in 10 week old mice induced a strong selection against c-Myc-deficient crypts within less than 4 weeks (Muncan et al., 2006). Interestingly, when investigating c-Myc-deficient crypts in more detail, it was found that cell number as well as cell size was reduced compared to Myc proficient crypts. By performing an AgNor staining (against the silver-stainable proteins of the nucleolar organizing regions), it was shown that biosynthetic activity was reduced in c-Myc-deficient crypt cells, leading to reduced growth rate and slower cell cycle kinetics. Although adhesion molecules and migration were not investigated in this study, it becomes clear that the functions of c-Myc greatly depend not only on the cell type but also on the developmental stage of the analyzed cell or organ.

3.2.4 conditional knockouts in other organs

Several other CKOs have been described for c-Myc, for example in pancreas (Bonal et al., 2008; Nakhai et al., 2008), kidney (Couillard and Trudel, 2009), liver (Baena et al., 2005; Li et al., 2006) and skin (Oskarsson et al., 2006; Zanet et al., 2005). Summarizing those reports one can say that c-Myc loss in vivo often has an impact on proliferation, differentiation and apoptosis, variable even within one organ, depending on the exact Cre transgene used, but maybe even more on the exact time point of induction. Also stem cells are frequently affected in the CKOs, which is most obvious during processes of regeneration, wound healing or development, while c-Myc seems rather dispensable for adult homeostasis. One example of very opposing results from literature can be found for the liver, where cell size and regeneration after hepatectomy were affected in one study using juvenile Mx-Cre mice (Baena et al., 2005), while another report shows successful proliferation after hepatectomy following AdCre vector injection in adult mice (Li et al., 2006). Similarly, contradictory results were observed by two groups using the K5-Cre to
delete c-Myc from skin. Mice in which the epidermis was c-Myc-deficient from embryonic day 14 on showed smaller cell size, decreased proliferation and stem cell amplification, leading to fragile skin, hair loss and greatly impaired wound healing (Zanet et al., 2005). In contrast, when deleted from skin in 3 weeks old mice, c-Myc was not required for homeostasis or chemically (TPA) induced hyperproliferation (Oskarsson et al., 2006). This shows how multifaceted and differentially regulated the *in vivo* effects of c-Myc can be.

### 3.3 targeting c-Myc in mouse tumorigenesis

Apart from the numerous CKO studies to investigate the physiological functions of c-Myc, several reports analyzed loss of Myc in non Myc-induced tumor models. As c-Myc is frequently elevated in various human malignancies, these reports, which are discussed below, give important insights for considering c-Myc as therapeutic target in cancer therapy.

#### 3.3.1 c-Myc in skin tumorigenesis

The same study that reported that c-Myc was dispensable for normal skin homeostasis also analyzed the effects of c-Myc loss on Ras-driven tumorigenesis (Oskarsson et al., 2006). In a first setting, c-Myc CKO (K5-Cre) mice were challenged with TPA to induce hyperproliferation, and interestingly no difference was found in c-Myc-deficient epidermis. In the second setting, double treatment with DMBA/TPA was used, which was shown to produce papillomas harboring one activating H-Ras mutation (Quintanilla et al., 1986). It is important to note that Myc and Ras were the first known example of oncogene cooperation during cellular transformation (Land et al., 1983). Strikingly, the number of DMBA/TPA induced papillomas was strongly reduced in mice with c-Myc-deficient skin. Furthermore, it was found that none of the tumors grown on mutant skin harbored the c-Myc deleted allele, suggesting that the tumors specifically selected ‘escaper cells’ and that c-Myc is absolutely required for Ras-driven tumorigenesis. More detailed analysis revealed that a key function of c-Myc during epidermal tumorigenesis was the repression of
the CDK inhibitor p21CIP1, which was strongly upregulated after c-Myc loss and could explain the resistance to papilloma formation.

3.3.2 c-Myc and the Wnt pathway

Apc (adenomatous polyposis coli) is part of the β-catenin destruction complex and an important mediator of the canonical Wnt signaling pathway. Mutation or loss of Apc leads to stabilization and accumulation of active β-catenin, which is a key event of colorectal cancer development (Korinek et al., 1997). As c-Myc is a well established transcriptional target of β-catenin/TCF (He et al., 1998), mouse models with truncated (Ap{\text{c}}^{\text{Min/+}}) or downregulated (Ap{\text{c}}^{\text{fl/fl}}AhCre{+}) Apc were employed to address the function of c-Myc on intestinal tumor formation (Sansom et al., 2007; Yekkala and Baudino, 2007). It was found that in Ap{\text{c}}^{\text{Min/+}}c-Myc{+/-} mice tumor incidence was greatly decreased by c-Myc haploinsufficiency, thereby increasing survival of the mice (Yekkala and Baudino, 2007). The polyps that formed were significantly smaller, which was only in part due to decreased proliferation and increased apoptosis, but mostly due to decreased production of angiogenic factors, like VEGF. In the Ap{\text{c}}^{\text{fl/fl}}Myc^{\text{fl/fl}}AhCre{+} setting, where almost 90% of the intestinal crypts were deficient for both genes after 4 days, it was found that loss of c-Myc reversed all perturbed phenotypes (proliferation, differentiation, migration) induced by Apc deletion (Sansom et al., 2007), while levels of nuclear β-catenin still remained high. Interestingly, microarray analysis showed that about 60% of the Wnt target genes in the intestines were Myc-dependent. The same Apc mouse model was used to study its function in liver, where Apc loss led to high levels of β-catenin and c-Myc, high proliferation and hepatomegaly (Reed et al., 2008). Surprisingly, c-Myc loss in the liver did not rescue any of the observed phenotypes, while loss of β-catenin did, highlighting the differences of Myc dependence in the Wnt pathway in specific organs.

3.3.3 whole body targeting of c-Myc in Ras-induced lung carcinomas

Another strategy was used to interfere with c-Myc signaling in the whole body, which is to interfere with its heterodimerization with Max. This is achieved via
expression of Omomyc, a dominant interfering Myc binding partner, that can prevent Max binding and Myc dependent transcriptional activation (for detailed analysis see Soucek et al., 1998; Soucek et al., 2002). Expression of Omomyc was able to reverse Myc-induced transformation in vitro (Soucek et al., 2002) as well as Myc-driven skin tumorigenesis in vivo (Soucek et al., 2004). In their latest study, Soucek and coworkers created an inducible Omomyc mouse, which expresses Omomyc in multiple tissues after administration of doxycycline (Soucek et al., 2008). Using this transgenic line, they investigated the function of c-Myc in tumor initiation and maintenance of a Kras-driven lung cancer mouse model (expressing the oncogenic Kras\textsuperscript{G12D}). Strikingly, interfering with Myc significantly reduced proliferation and blocked formation of lesions. When expressing Omomyc for only one week after 6 weeks of tumor growth, a marked shrinkage of lesions was observed and remaining lesions were highly apoptotic. Finally, after 18 weeks of tumor growth when mice had developed multiple lesions and some highly vascularized adenocarcinomas, Myc interference again induced shrinkage after only 3 days, with significant reduction of proliferation after 6 days of Omomyc expression. After 28 days of expression, lungs appeared to be free of obvious tumors, showing that Myc function is indispensable for all stages of Kras\textsuperscript{G12D}-driven lung tumorigenesis. Importantly, the observed side effects during 4 weeks of Omomyc expression were tolerable and general health of the mice was not affected. Rapid turning-over organs, like skin, testis and intestines that were significantly affected by Myc inhibition, all recovered within one week after ending Omomyc expression.

### 3.3.4 c-Myc in Notch-induced mammary tumorigenesis

Besides the above discussed tumor models in skin, intestines, liver and lung, there is one single study investigating the loss of c-Myc in mammary tumorigenesis, induced by the constitutively active Notch1 intracellular domain (IC) (Klinakis et al., 2006). This approach used MMTV-Notch1\textsuperscript{IC} transgenic mice which develop lactation-dependent regressing and non-regressing tumors that showed increased Myc expression as well as high microarray profile similarity with MMTV-Myc induced tumors. The MMTV-Notch1\textsuperscript{IC} transgenic mice were further crossed with
myc\textsuperscript{fl/fl}WapCre mice to analyze whether the upregulated Myc levels in Notch-induced tumors were functionally relevant. The authors observed that development of regressing tumors was almost completely prevented upon Myc loss, and non-regressing tumors (observed from 7 to 15 months) showed a highly increased latency and a reduced incidence in Myc-deficient mice. In addition, all microlesions or eventual tumors that were examined in Myc-deficient mice were shown to stem from ‘escaper’ cells that had retained Myc, and no recombined allele was detected in any of the tumors. The authors concluded from this that Myc is indispensable for Notch-induced mammary tumorigenesis and further showed that Myc is a direct transcriptional target of Notch1. One drawback of this study is that two hormonally controlled Cre lines are combined, whose onset cannot be regulated by the investigator; thus, the question of Myc function after establishment of the tumor could not be addressed in this study. Still, the effect is particularly interesting as the authors also detected a correlation between expression of the two genes in patient samples of breast carcinomas. Given the large number of signaling pathways that mediate directly or indirectly via c-Myc, like Wnt, ER and ErbB2, it will be interesting to investigate the impact of Myc loss in cancer models depending on those pathways.
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V. Breast Cancer Research Review

Review
Key signalling nodes in mammary gland development and cancer

Myc
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Abstract
Myc has been intensely studied since its discovery more than 25 years ago. Insight has been gained into Myc's function in normal physiology, where its role appears to be organ specific, and in cancer where many mechanisms contribute to aberrant Myc expression. Numerous signals and pathways converge on Myc, which in turn acts on a continuously growing number of identified targets, via transcriptional and nontranscriptional mechanisms. This review will concentrate on Myc as a signaling mediator in the mammary gland, discussing its regulation and function during normal development, as well as its activation and roles in breast cancer.

Introduction
Since the early 1980s, numerous investigations have focused on c-Myc to explore its role in normal organ physiology, as well as in tumor biology [1,2]. The focus of the present review, c-Myc (hereafter referred to as Myc), is the cellular homolog of the avian retroviral oncogene v-myc and, together with N-myc and L-myc, comprises the family of myc proto-oncogenes. The half-lives of Myc mRNA and protein are short, allowing for tight and rapid regulation of Myc levels, which occurs via numerous transcription factors (TFs) and signaling pathways. Proteins that directly bind the promoter or indirectly influence promoter activity have been reviewed recently [3]. To provide some insight into the complexity of Myc regulation, we will mention a few of the factors and pathways that impact on its expression, many of which were shown to be essential during mammary gland development [4].

The myc promoter contains TF binding sites for Myc (auto-suppression), estrogen receptor (ER) alpha, T-cell factor (TCF) 4, Notch/C promoter-binding factor 1 (Cbf1), E2F, Fos/Jun, signal transducer and activator of transcription (Stat) 3, NF-xB, Smads and others. TFs that occupy or regulate the myc promoter without specific binding sites include p53, CCAAT/enhancer binding protein beta, and Stat5. Moreover, numerous signaling pathways that are frequently deregulated in human cancer influence myc expression; for example, rat sarcoma (Ras)/extracellular signal-related kinase (Erk) and phosphoinositide 3-kinase (P3K)/serine/threonine kinase Akt (Akt). Post-translational modifications of Myc include phosphorylation, ubiquitinylation and acetylation, and their effects on Myc activity have been reviewed [5].

The Myc protein is a basic helix–loop–helix TF that must heterodimerize with the abundantly expressed Max to regulate transcription. Myc–Max dimers bind to hexameric DNA sequences (E-box) and activate transcription by recruiting multiple coactivators [1]. In contrast, when dimerized with basic helix–loop–helix proteins such as Mad or Mnt, Max binds to E-boxes but represses transcription. Myc can also act as a transcriptional repressor via different mechanisms, often involving interaction with Miz1 (for reviews of Miz1 transcriptional activity, see [1,6]). It is now well accepted that Myc acts as a relatively weak activator of RNA polymerase II-driven transcription for a large set of target genes, thereby affecting cell cycle, cell growth and metabolism, cell death, adhesion, angiogenesis and other functions (to date, almost 1,700 targets on Myc Cancer Gene [7,8]). Furthermore, Myc affects RNA polymerase I- and II-mediated transcription [1], thus regulating ribosome biogenesis and translation. Nontranscriptional roles for Myc in DNA replication and in translation have also been reported recently [9].

Considering the large number of receptors, hormones, paracrine factors and other signaling molecules that can

Akt = serine/threonine kinase Akt; APC = adenomatosis polyposis coli; Cav1 = Caveolin1; Cbf1 = C promoter-binding factor 1; Cdk = cyclin-dependent kinase; ER = estrogen receptor; Erk = extracellular signal-related kinase; K14 = Keratin 14; MMTV = mouse mammary tumor virus; MIB1/TOM = MMTV-HTA/TatO-MYC; NF = nuclear factor; NFκB = Notch intracellular domain; Nrg3 = Neuregulin3; P3K = phosphoinositide 3-kinase; Ras = rat sarcoma; SC = stem cell; sFRP1 = secreted Frizzled-related protein 1; Stat = signal transducer and activator of transcription; TCF = T-cell factor; TF = transcription factor.
impact on Myc levels, it is likely that Myc has a variety of functions throughout normal mammary gland development, downstream of one or more of these inputs. To date, however, there are only a few studies on the physiological role of Myc in the mammary gland. In this review we present what is known about Myc from transgenics and conditional knockout models, and also include indirect evidence implicating Myc based on results from other studies. We shall discuss the potential inputs activating Myc during development and the ensuing outputs of Myc activity. A summary of the discussion on the role of Myc in normal development is shown in Figure 1.

Embryogenesis
Development of the mammary gland begins at embryonic day 10 as an appendix of the ventral skin, with formation of the milk line followed by appearance of placodes [10]. The ErbB4 ligand, Neuregulin3 (Nrg3), was identified as a specification signal for placode formation. Based on this, Keratin14 (K14)-Nrg3 transgenic mice expressing Nrg3 throughout the basal layer of the epidermis, including the stem and progenitor cells, were investigated [11]. Ectopic Nrg3 expression resulted in hyperplastic epidermis and formation of supernumerary placodes. Interestingly, the skin of K14-Nrg3 mice displayed increased Myc expression and decreased levels of α-case integrin and β1-integrin, which are adhesion receptors highly expressed in adult mammary stem cells (SCs) [12]. While the direct stimulus of Myc expression is unknown, it seems to be an important mediator of the phenotype observed in K14-Nrg3 mice, as strong similarities were found in a K14-Myc model where Myc is activated in the epidermis [11]. Nrg3 therefore possibly has a role in promoting mammary lineage commitment and in the regulation of SC fate via Myc.

Mammary stem cells
While these results suggest a role for Myc in embryonic development, its role in adult mammary SCs has not yet been analyzed. A function for Myc in mammary SCs seems likely, however, based on its role in other well-characterized models [1,13]. In the hematopoietic system, the balance between SC self-renewal and differentiation is controlled by Myc levels, which in turn regulate expression of adhesion molecules such as N-cadherin and β1-integrin [14]. In addition, the Wnt and Notch signaling pathways have been proposed to play important roles in mammary SCs [13], and their effector proteins – β-catenin/TCF and Notch intracellular domain (NICD)/Cbf1, respectively – each have binding sites on the Myc promoter [3]. Furthermore, a transgenic model expressing stabilized β-catenin in basal cells, which are believed to contain the SC population [12], displayed upregulation of Myc [15]. Finally, the HCl1 mammary epithelial cell line, which has SC-like properties, might be an interesting model to explore Myc function, since Myc levels are downregulated when these cells are induced to differentiate [16].

Puberty and pregnancy
The steroid hormones estrogen and progesterone, as well as the prolactin receptor binding peptides prolactin and placental lactogen, dominate the extensive developmental changes occurring during puberty and pregnancy [4,17]. Both estrogen and progesterone are able to directly stimulate Myc expression via an estrogen response element [18] and a progesterone receptor regulatory element [19]. While Myc is expressed at low levels in prepubertal and virgin glands, it reaches its highest expression levels between days 6.5 and 12.5 of pregnancy, after which the RNA level slowly returns to baseline until parturition [20].

Interestingly, Myc was shown to be directly downstream of estrogen and progesterone in breast cancer cells, stimulating their proliferation [21,22], but one of the major differences between normal breast and malignant breast is that the estrogen- and progesterone receptor-positive cells do not proliferate during normal development. Instead, by producing paracrine mediators, estrogen and progesterone stimulate proliferation of neighboring cells via amphiregulin [23] and Wnt4 [24], respectively. Other growth factors, such as epidermal growth factor or receptor activator of NF-κB ligand, have also been shown to act as paracrine mediators during mammary gland development [25]; and importantly, Wnt, epidermal growth factor and receptor activator of NF-κB ligand might all affect Myc levels directly or indirectly [9].

Although no study has yet addressed the contribution of Myc to pubertal development and early pregnancy, it is very likely that Myc is induced via paracrine signals, and, at least early in pregnancy when Myc levels are highest, Myc might promote proliferation. Importantly, different Myc targets such as cyclin-dependent kinase (Cdk) 4, nucleoplasmin and nucleolin are also highly expressed at this time [20] – prompting us to speculate that Myc might not only have a direct role in proliferation, but also in the synthesis of ribosomal components required for rapid growth during pregnancy.

A transgenic model revealed why it is important for Myc levels to decrease, starting at 12.5 days of pregnancy, and to remain low until parturition. Using a doxycycin inducible model (MMTV-tTA/TetO-MYC (MTB/TOM)) [26], it was shown that transient overexpression of Myc between days 12.5 and 15.5 of pregnancy induced a lactation failure [27]. Abnormal Myc expression was shown not only to induce proliferation, but also to promote precocious Stat5 activation and differentiation, followed by premature involution, triggered by milk stasis. Decreased levels of Caveolin1 (Cav1), a direct target of Myc repression [7], were shown to be responsible for the phenotype. Cav1 is a negative regulator of Janus kinase2–Stat5 signaling, and Cav1−/− mammary epithelial cells show hyperactivation of Stat5 and spontaneous milk production [28], similar to what is observed when Myc is elevated late in pregnancy.
Ins and outs of the ‘black box’ MYC during normal mammary gland development. The diagram displays the models (italic, top) used to investigate the various inputs and outputs of Myc (green boxes). Speculations based on other model systems that have not yet been shown in the mammary gland are presented in red. Inputs are signaling molecules that are known or suggested to impact on Myc levels; inputs are not applicable (n.a.) in transgenic models with genetically deregulated Myc levels. The outputs are, where available, direct targets of Myc transcriptional activity and general biological functions described for Myc at the specific developmental stage (italic, bottom). During embryogenesis, transgenic expression of Neuregulin3 (Nrg3), a major factor controlling mammary placode development, induced high Myc levels, thereby changing the proliferative and adhesive properties of cells [11]. The speculated role of Myc in mammary stem cells (SCs) is mostly based on data from hematopoietic SCs and the known importance of Wnt and Notch pathways in other SC types [13]. Myc’s role during puberty and early pregnancy has not yet been analyzed, but as various steroids and paracrine factors can induce its expression [3] Myc might play a role in promoting proliferation and cell growth via its numerous cell cycle and translation-related targets. A transgenic mouse model (MMTV-rtTA/TetO-MYC (MTB/TOM)) revealed that Myc overexpression during late pregnancy leads to precocious proliferation and differentiation via repression of Caveolin1 (Cav1) and signal transducer and activator of transcription (Stat5) hyperactivation [27]. Despite its low levels during lactation, Myc has an important role in mRNA translation, as shown in our own laboratory using mammary glands conditionally lacking Myc (Myc-CKO) [29]. Finally, in Sox9 conditional knockout (CKO) mice it was shown that increased Stat3 activation leads to accelerated apoptosis via high levels of Myc, suggesting a direct role for Myc downstream of Stat3 in involution [31]. More detailed discussion can be found in the text, K14, Keratin14; KO, knockout; N-Cad, N-cadherin.

These results demonstrate an important characteristic of Myc – namely, the effects of Myc are dependent upon the developmental stage of the mammary gland. Myc overexpression between 12.5 and 15.5 days of pregnancy was necessary and sufficient to induce the observed phenotype, while overexpression during other short intervals (for example, 9.5 to 12.5 days) did not result in lactation failure [27]. Deregulated Myc therefore leads to a premature decrease in Cav1, thereby removing its restraining influence on prolactin receptor–Janus kinase2–Stat5 signaling.

**Lactation**

In many cell types Myc is downregulated when cells undergo terminal differentiation. Indeed, Myc RNA levels do drop during lactation to below those levels found in the virgin gland [20]; however, the molecular reason for this dramatic decrease is not known. The mammary gland as a milk factory produces immense amounts of lipids, lactose and proteins, and most of its energy is devoted to milk component synthesis. Considering the importance of Myc in energy and glucose metabolism as well as ribosome biogenesis and translation [1], it is possible that, even despite its low levels, Myc has an essential function during lactation.

Indeed, data from our laboratory revealed a novel role for Myc in the mammary gland using a conditional knockout approach. In c-myc<sup>cre</sup>/WAP<sup>cre</sup> (whey acidic protein)<sub>Cre</sub> mice, loss of Myc occurs exclusively in luminal alveolar cells starting from mid-pregnancy. We show that milk production was reduced in Myc mutant mothers, while milk composition was unchanged between wild-type and mutant mothers [29]. Electron microscopy revealed that there were less secretory vesicles budding from the endoplasmic reticulum in lactating mutant cells, suggesting a decreased protein synthesis. In polysomal fractionation experiments we found that translation efficiency was generally decreased in lactating, Myc-deficient mammary glands. Furthermore, we observed reduced expression levels of ribosomal proteins and RNA, as well as proteins involved in translation and ribosome biogenesis. Although compensation by N-Myc or L-Myc cannot be
excluded, neither was found upregulated in c-Myc mutant glands. These results highlight Myc’s importance for mammary gland function even when endogenous levels are low.

**Involution**

The impact of Myc on apoptosis has been widely studied in many systems [2]. In the mammary gland, the high levels of apoptosis during the first phase of involution are promoted by the leukemia inhibitory factor–Stat3 axis [30]. Compared with its low expression in lactating glands, higher Myc levels are detected during involution [20]. Importantly, a role for Myc during the first apoptotic phase was uncovered in mice with a conditional deletion of Socs3 (Socs3−/−VAPIcicre), a negative regulator of leukemia inhibitory factor–Stat3 signaling [31]. Socs3-deficient glands displayed accelerated apoptosis accompanied by elevated levels of p-Stat3 and Myc, which is a direct Stat3 target gene.

To further analyze Myc’s function in apoptosis, the doxycycline-inducible MTB/TOM model described above [26] was used, giving more evidence for a direct role of Myc in involution. Overexpression of Myc prior to forced weaning caused a dramatic acceleration of involution, accompanied by increased apoptosis and high levels of the pro-apoptotic proteins Bax, E2F-1 and p53, which have all been described as direct or indirect Myc targets.

The conclusions based on those two models suggest that Myc acts as a central mediator of apoptotic signaling in the mammary gland, being a direct target of Stat3 and inducing expression of pro-apoptotic genes.

**Background on Myc in breast cancer**

Alterations in Myc have been found in many types of tumors. At the genomic level these include gene amplification, chromosomal translocations and point mutations. Furthermore, Myc is regulated by multiple signals that control promoter activity, transcriptional elongation and translation, as well as by post-translational modifications that control Myc’s transcriptional targets as well as protein stability. Since most tumors have numerous alterations in signaling cascades, Myc is likely to be deregulated by some mechanisms in most cancers.

Considering breast cancer, amplification is the most commonly described alteration. The MYC amplicon on chromosome 8q23-24 was one of the first consistent genetic alterations found [32]. Results from a meta-analysis of breast tumors yielded a frequency of 15.7% for the MYC amplicon, with a range of 4 to 52% depending on the study [21,33]. MYC amplification is found in a high proportion of tumors with Brca1 alterations, as well as in Erx-negative, basal-like tumors [34,35]. Despite intense screening efforts, point mutations of Myc have not been described in breast or other carcinomas [36]. Other mechanisms promoting increased Myc levels, however, have been found. The ubiquitin ligase F-box and WB repeat domain containing 7, which catalyzes polyubiquitination of Myc and ensuing degradation, is often mutated or downregulated in breast cancer [37]. Moreover, the de-ubiquitinating enzyme ubiquitin-specific protease 28 – which antagonizes F-box and WB repeat domain containing 7, thereby stabilizing Myc – has been found overexpressed in a small panel of breast tumors [38]. In addition to these breast tumor-specific alterations, it is possible that Myc is de-regulated in most breast tumors since the normal tight control that is exerted on Myc at multiple levels is impaired in essentially all cancer cells.

What is the output of deregulated Myc in breast cancer? Myc levels respond to proliferative and anti-proliferative stimuli, and many reported Myc target genes, such as cyclin D3, Cdk4 and the Cdk inhibitor p21Cip1, are important regulators of proliferation [2]. A major mechanism underlying Myc’s role in breast cancer is activation of cyclin E–Cdk2 via repression of p21Cip1 [21]. Myc deregulation not only impacts on proliferation, but on various other processes like survival and apoptosis.

Here we would like to mention Myc and translational control, since we have recently shown an important role for Myc in translation also during mammary gland development [29]. Generation of the Eμ-Myc B-cell leukemia model in mice with heterozygosity in the gene encoding the L24 ribosomal protein restored normal levels of protein synthesis in the leukemic cells, thereby suppressing Myc’s oncogenic potential [39]. These results show that, in addition to proliferative effects, abnormal Myc activation also deregulates protein synthesis, which in this model is necessary for oncogenesis.

Considering the broad-ranging effects of Myc, the outcome of its activation in breast cancer is likely to be dependent upon the cellular context. Indeed, using a siRNA approach to knockdown Myc in a panel of breast cancer cell lines, and combining this with a genomic and phenotypic analysis, revealed that selectively regulated target genes in each cell line were responsible for the differential effects resulting from Myc loss. A comprehensive list of potential Myc targets in the BT-474, MCF-7 and MDA-MB-231 breast cancer cell lines can be found in Cappellen and colleagues’ study [40]. A summary of the discussion on the role of Myc in cancer is shown in Figure 2.

**Myc and mammary cancer**

Myc was the first oncogene tested for mammary tumor-forming potential using the mouse mammary tumor virus (MMTV)-long terminal repeat to drive its expression. Tumor incidence in MMTV-Myc transgenic females was high; however, the kinetics of tumor appearance suggested that Myc expression alone was not sufficient to induce cancer [41]. Indeed, double transgenics expressing Myc and mutant Hras showed more rapid mammary tumor development [2]. More recently, it was shown that Myc induction using the
Aberrant Myc expression causes mammary cancer. Myc is deregulated in most mammary tumors by multiple mechanisms, including gene amplification, or aberrant expression due to alterations in signaling pathways that influence Myc RNA or protein levels as well as its transcriptional activity. Each of the indicated proteins or pathways impacts on Myc expression or activity in mammary cancer. Specifically, the Notch and Wnt pathway effectors, Notch intracellular domain/C promoter-binding factor 1 and β-catenin/T-cell factor, respectively, as well as estrogen receptor alpha (ERα), bind to the Myc promoter, thereby stimulating transcription. Dysregulation of transforming growth factor beta (TGFβ) and Brca1 in mammary cancer has been reviewed recently [34]. TGFβ, via Smads, suppresses Myc expression, while Brca1, which is frequently deregulated in ERα-negative, basal-like breast cancer, normally blocks Myc transcriptional activity. The ubiquitin-specific protease ubiquitin-specific protease 28 (USP28) was found overexpressed in breast tumors [38] and stabilizes Myc via antagonizing F-box and WD repeat domain-containing 7 (FBW7), which is frequently lost or mutated in breast tumors [37]. Finally, ErbB2 activation, which is also regulated by ERα, stimulates pathways like rat sarcoma/extraordinary signal-related kinase (Ras/Erk) and phosphoinositol 3-kinase/protein kinase B (PI3K/Akt) that influence Myc RNA and protein levels. See text for further details. Myc is an activator of RNA polymerase II-driven transcription for multiple target genes [2] and also affects RNA polymerase I and III-mediated transcription, thus regulating ribosome biogenesis and translation. In cancer cells, the outcome of deregulated Myc will be wide-ranging considering that Myc influences the cell cycle, protein synthesis, cell growth and metabolism, cell death, genomic instability, tumor-induced angiogenesis, adhesion, as well as other cellular functions. This is exemplified by examining the effects of Myc knockdown in breast cancer cell lines, where a genomic and phenotypic analysis revealed that selectively regulated target genes in each cell line were responsible for the differential effects resulting from Myc loss [40].

Based on the observation that mammary tumors in the MMTV-Nc transgensics presented elevated Myc, its role in Notch transformation was examined in mice with floxed Myc alleles [43]. Conditional ablation of Myc using the WAPCre transgene revealed that Myc was indispensable for development of Nc-driven mammary tumors. This contribution of Myc to Notch-induced tumorigenesis is interesting especially when comparing it with Wnt pathway-driven models (see below). Moreover, Myc was shown to be a direct target of the Notch pathway, since a complex of Nc and Cbf1 was detected on the Myc promoter. The Cbf1 binding site on the human Myc promoter is conserved, and immunohistochemistry revealed that there was a significant correlation between high Myc levels and Nc in human breast tumors [43]. It is intriguing that coexpression of Jagged ligand and Notch receptors is found in triple-negative breast tumors [44], a subgroup that also has high Myc activity [46].

**Myc and the Wnt pathway**

Wnt1 was the first identified oncogene activated by MMTV insertional mutagenesis. Wnt-mediated activation of the canonical pathway leads to β-catenin stabilization, TCF binding and transcriptional activation of Myc. Mammary tumors arising in Wnt1 transgenics [47] and models driven
Introduction: Review

by a β-catenin-stabilized mutant [15] show elevated levels of Myc. Human breast tumors, unlike colon cancer, do not possess Wnt pathway-activating mutations. Derepression of Wnt signaling appears to occur by autocrine mechanisms, however, since multiple Wnt ligands and Frizzled receptors are coexpressed [48], and the negative Wnt pathway regulator - secreted Frizzled-related protein (sFRP1) – is often absent [49]. A positive feedback loop has also been described for Myc and the Wnt pathway. In telomerase-immortalized, Myc-transformed human mammary epithelial cells, Myc was shown to repress sFRP1 and Dickkopf 1, another negative pathway regulator, thereby contributing to activation of canonical Wnt signaling [50]. Along the same lines, Myc knockdown in MDA-MB-231 tumor cells increased Dickkopf 3 expression [40].

What is Myc’s role in tumors induced by Wnt pathway activation? The dependency of Wnt-driven mouse mammary tumors on Myc expression has not been tested. In other tumor models driven by adenomatosis polyposis coli (APC) loss, the importance of Myc has been examined using organ-specific Cre recombinase-mediated deletion of floxed Myc alleles. In the intestines, Myc deletion reversed the tumor phenotype induced by APC loss, and it was shown that the majority of Wnt targets in the intestines were Myc dependent [51]. In striking contrast, Myc deletion had no affect on the phenotype of APC loss in the liver, where most Wnt target genes were β-catenin dependent but Myc independent [52].

These two studies [51,52] reveal that the importance of Myc in a particular tumor model is very specific and can differ as already discussed for the normal organ-specific Myc functions. As discussed above, deregulated Myc might enforce autocrine Wnt pathway activity in human tumors by repressing negative regulators such as sFRP1. Other potential roles for Myc have not been examined; however, blockade of the Wnt pathway usually results in lowering Myc levels. Stable expression of sFRP1 in MDA-MB-157 and MDA-MB-231 breast tumor cell lines blocks proliferation of both cell lines, and Myc RNA was decreased in the former [53] while Myc protein was lower in the latter [49]. In a panel of breast cancer cell lines, siRNA-mediated knockdown of Dishevelled, an essential mediator of Wnt signaling, led to lower Myc and decreased proliferation in most cell lines [54].

In summary, the current data suggest that, in mouse and human mammary tumors with constitutive Wnt signaling, Myc levels are elevated and might have a role in transformation.

Myc and ErbB2

Amplification of ERBB2 leading to receptor overexpression is found in 20 to 25% of primary breast tumors. In these tumors, constitutive ErbB2 activation stimulates numerous intracellular signaling pathways including Ras/Erk and PI3K/Akt, both of which impact on Myc transcription and protein stability. The role of Myc has been examined in the ErbB2-overexpressing SKBr3 and BT-474 breast tumor cell lines. Treatment of both with the ErbB2-specific antibody trastuzumab caused a cell cycle block that was accompanied by a decrease in PI3K/Akt pathway activity, and by downregulation of Myc and D-type cyclins [55]. Interestingly, ectopic expression of Myc in SKBr3 cells partially rescued the cells from functional ErbB2 inactivation [56], pointing to the importance of Myc as an ErbB2 effector.

Myc and estrogen receptor alpha

In the normal breast, both rodent and human, a major role of the ERα-positive cells is to act as sensors to relay a proliferative signal to neighboring cells. In contrast, many breast tumor cells are ERα-positive, and they have not only acquired the potential to proliferate in response to steroid hormones but are also dependent upon them for survival [21,57]. Since Myc is an ERα target, it is important to understand whether Myc has a role in acquisition of this phenotype. Moreover, since patients whose tumors are ERα-positive are treated with anti-estrogen therapy, Myc’s role in response or resistance is also of high interest. Unfortunately, consistent clinical data relating MYC amplification or expression levels to endocrine therapy response are not available [21]. Overexpression of ErbB2 has been correlated with de novo and acquired endocrine resistance [57], however, and Myc is an ErbB2 effector.

The role of Myc in ERα signaling has been well characterized in the MCF-7 breast tumor cell line [21]. Myc RNA increases rapidly in response to estrogen treatment of these cells, and Myc knockdown impairs the ability of estrogens to stimulate proliferation. Furthermore, Myc overexpression in cells arrested by ERα antagonists overcomes the proliferative block. Interestingly, adaptation of MCF-7 cells to growth in estrogen-deprived medium is associated with upregulation of ERα-regulated target genes, including Myc [58], suggesting a mechanism whereby Myc maintains an important role in proliferation and survival even in the absence of ERα activity. It should also be mentioned that ERα cross-regulates ErbB2, which in turn impacts on Myc via activation of downstream signaling pathways [57].

From transcriptome and network analyses, the close link between Myc and ERα signaling has become even more apparent. MCF-7 cells show a high level of overlap between ERα-regulated and Myc-regulated genes. Indeed, more than 50% of the estrogen-responsive genes are also Myc targets [59]. Moreover, a meta-analysis of transcriptional and pathway data, carried out on primary breast tumors, revealed that Myc activity is elevated in ERα negative, basal-like breast cancers, as measured by target gene levels [46].

In summary, these data led to the proposal that elevated Myc activity present in ERα-negative breast tumor cells mimics the activity of estrogen on ERα-positive breast tumor cells [46,59].
Myc as a prognostic, predictive or therapeutic target in breast cancer

Considering that Myc deregulation is so common in breast cancer, Myc has been examined as a prognostic factor and as a predictive factor. Indeed, MYC amplification is associated with aggressive clinical features including high grade and lymph node positivity, and correlates with poor patient outcome [34]. A major interest in breast cancer is the use of genetic alterations to categorize patients into treatment groups; a good example being detection of the ERBB2 amplicon for trastuzumab treatment. Gene expression signatures are also being generated in order to provide predictive data on patient response to standard chemotherapeutics and to targeted therapy. In one study of chemotherapy-treated breast cancer patients, an attempt to correlate Myc pathway activity with response yielded inconsistent results. The group with Myc and Rae pathway activation had a high percentage of responders, while patients whose tumors had Myc and E2F pathway activity responded poorly [60]. These results underscore the difficulty of using Myc levels alone as a predictive or prognostic factor, and emphasize the fact that the cellular context of Myc expression and activity determine the outcome.

Approaches to target Myc activity in tumors are also under consideration [34,61]. Although targeting Myc is appealing, there are many difficulties associated with altering transcription factor activity. Considering the variety of kinase inhibitors currently available, it is worth considering their use in breast tumors with Myc deregulation. Based on the molecular concepts analysis [62] of the Myc pathway activation signature in cancer, and on the identification of the genes downregulated in MCF-7 cells treated withwortmannin and LY-294002, there is reason to believe that PI3K inhibitors might be especially potent in breast cancers with high Myc activity. As discussed [46], ERα-negative, basal-like breast tumors with high Myc activity might be particularly susceptible to PI3K inhibitors.

Conclusion

Clearly, there are numerous signaling events in the development of the mammary gland that can be mediated, at least in part, via Myc. Using transgenic models, functions for Myc – some potential, others data based – have been discussed for embryonic development, pregnancy, lactation and involution. Furthermore, results from other model systems suggest that Myc could play a role in stem cell fate, as well as during early pregnancy, where Myc expression levels are highest in the normal gland. Even more efforts have been made to investigate Myc’s role in transformation, as deregulation of Myc via amplification, overexpression or stabilization of the protein is a frequent event in breast cancer. Signaling pathways implicated in breast cancer, such as ERα, ErbB2, Notch and Wnt, all contribute to aberrant Myc levels or activity. The challenge for future studies will be to reveal the suitability of targeting Myc for breast cancer treatment, either by direct inhibition or by indirectly targeting another pathway.

Competing interests

The authors declare that they have no competing interests.

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17. Hovey RC, Trott JF, Vonderhaar BK: Establishing a framework


Aims of the Work

c-Myc plays a central role in signaling pathways frequently deregulated in breast cancer and might especially gain importance in the basal, poor prognosis subtype, where high pathway activation of c-Myc is found. Furthermore, transgenic models revealed that c-Myc overexpression affects normal mammary gland development during embryogenesis, pregnancy/lactation and involution. However, before this study the physiological functions of c-Myc had not been addressed using knockout mice.

We investigated this open question by using a conditional knockout approach, crossing c-myc<sup>fl/fl</sup> mice to mice heterozygous for the WAP<i>Cre</i> transgene. Thereby we wished to identify the phenotype resulting from c-Myc loss during first pregnancy, and its effects on subsequent pregnancies. In addition, we aimed at identifying the mechanisms behind the observed phenotype(s) by performing different experiments with the mammary glands of mutant and wild type mice. When we observed that c-Myc mutant mice showed a decreased nursing efficiency, we performed detailed analysis of mutant milk samples as well as histology of the glands, revealing that milk production was reduced in mutant mothers. In order to obtain statistically significant results, we carried out image analysis and quantified alveolar versus total organ area, using Definiens software. The remaining challenge was to identify the targets or pathways that were affected by c-Myc loss, as it is known that c-Myc is a very broad, but weak transcriptional regulator of various cellular processes. This was accomplished by performing electron microscopy studies and establishing a polysome fractionation of mammary gland lysates. Our hypothesis of reduced translation efficiency was affirmed by qPCR results showing that components involved in ribosome biogenesis and translation were reduced in mutant glands. One surprising finding was the differential effect on mRNAs whose protein products are involved in milk synthesis, as compared to mRNAs of housekeeping genes. Finally, we performed a ‘cleared fat pad’ mammary transplantation assay to investigate the effect of c-Myc loss on progenitor cells.
RESULTS

Research article

c-Myc affects mRNA translation, cell proliferation and progenitor cell function in the mammary gland

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Abstract

Background: The oncoprotein c-Myc has been intensely studied in breast cancer and mouse mammary tumor models, but relatively little is known about the normal physiological role of c-Myc in the mammary gland. Here we investigated functions of c-Myc during mouse mammary gland development using a conditional knockout approach.

Results: Generation of c-Mycfl/fl mice carrying the mammary gland-specific WAPcRE transgene resulted in c-Myc loss in alveolar epithelial cells starting in mid-pregnancy. Three major phenotypes were observed in glands of mutant mice. First, c-Myc-deficient alveolar cells had a slower proliferative response at the start of pregnancy, causing a delay but not a block of alveolar development. Second, while milk composition was comparable between wild type and mutant animals, milk production was reduced in mutant glands, leading to slower pup weight-gain. Electron microscopy and polysome fractionation revealed a general decrease in translational efficiency. Furthermore, analysis of mRNA distribution along the polysome gradient demonstrated that this effect was specific for mRNAs whose protein products are involved in milk synthesis. Moreover, quantitative reverse transcription-polymerase chain reaction analysis revealed decreased levels of ribosomal RNAs and ribosomal protein-encoding mRNAs in mutant glands. Third, using the mammary transplantation technique to functionally identify alveolar progenitor cells, we observed that the mutant epithelium has a reduced ability to repopulate the gland when transplanted into NOD/SCID recipients.

Conclusion: We have demonstrated that c-Myc plays multiple roles in the mouse mammary gland during pregnancy and lactation. c-Myc loss delayed, but did not block proliferation and differentiation in pregnancy. During lactation, lower levels of ribosomal RNAs and proteins were present and translation was generally decreased in mutant glands. Finally, the transplantation studies suggest a role for c-Myc in progenitor cell proliferation and/or survival.

See related minireview by Evan et al: http://jbiol.com/content/8/8/77
Background

The oncoprotein c-Myc is a basic helix-loop-helix transcription factor implicated in multiple cellular processes, including proliferation, differentiation, metabolism, and apoptosis (reviewed in Eilers and Eisenman [1]). c-Myc regulates RNA polymerase II (Pol II) driven transcription of a large set of targets [2-4] and has been reported to have effects on global chromatin modification [5]. Furthermore, c-Myc stimulates RNA Pol I [6,7] and Pol III [8,9] mediated transcription, thus linking it to ribosome biogenesis and translation. In addition, c-Myc has been implicated in mitochondrial biogenesis [10] and global miRNA expression [11]. Recently, non-transcriptional effects of c-Myc on DNA replication [12] and translation progression [13] have also been described.

Deregulated levels of c-Myc, resulting from amplification, translocation, transcriptional, translational as well as other mechanisms have been observed in numerous human tumors (reviewed in Vita and Henriksson [14]). In breast cancer, c-Myc overexpression occurs in >50% of primary tumors [15] and has been reported to correlate with poor prognosis [16]. The use of transgenic mouse models has helped to analyze c-Myc-induced mammary tumorigenesis [17-19], but little is known about the normal physiological function of c-Myc in the mammary gland.

A number of studies have described different roles for c-Myc in other organs. The full knockout of c-Myc is embryonic lethal [20,21], due to its indispensable function in the placenta and the hematopoietic system [22,23]. Several conditional mouse models expressing Cre recombinase under different promoters have been generated in order to delete c-Myc in skin [24,25], liver [3,26,27], pancreas [28,29], intestines [30,31], and bone marrow [32,33]. Taken together, the results revealed various organspecific roles for c-Myc in controlling development and regeneration, cell size or number and stem cell differentiation and maintenance. Each report is of interest, not only for deciphering physiological functions of c-Myc, but also when considering c-Myc as a therapeutic target in human cancer.

The mammary gland is a convenient model for developmental studies, as it goes through repeated cycles of proliferation, differentiation and apoptosis during puberty and pregnancy. The gland of a mature virgin female consists of two compartments, a ductal epithelial network and the stroma or mammary fat pad. Upon hormonal stimulation in pregnancy, bursts of proliferation followed by differentiation allow the gland to convert into a milk-synthesizing machine. To study the role of c-Myc in the mammary gland, a conditional approach using the Cre-loxP system was employed. Whey acidic protein (WAP)Cre transgenic mice were used to recombine the LoxP-flanked c-myc locus in luminal alveolar cells starting at mid-pregnancy and throughout lactation. Following loss of c-Myc in the mammary gland, three main phenotypes were observed. At the start of pregnancy, c-Myc-deficient alveolar cells were impeded in their proliferative response resulting in a delayed ability to differentiate. Moreover, mutant glands displayed lower expression levels of ribosomal RNA and proteins as well as a general decrease in translation. Finally, the mutant mammary epithelium had a reduced ability to grow when transplanted into mammary fat pads. These results suggest that c-Myc has multiple roles in the mammary gland, affecting proliferation, biosynthetic capacity, and progenitor cell proliferation and/or survival.

Results

WAPCre-mediated ablation of c-Myc in the mammary gland

To study the role of c-Myc in mammary gland development, we used a conditional approach, crossing c-myc<sup>fl</sup> mice [21] to WAPCre transgenic mice [35]. The generated offspring will be referred to as wild type (WT, c-myc<sup>fl/ fl</sup>;WAPCre<sup>+</sup> or c-myc<sup>fl/ fl</sup>;WAPCre<sup>-</sup>), heterozygous (c-myc<sup>fl/ fl</sup>;WAPCre<sup>+</sup>) and mutant (c-myc<sup>fl/ fl</sup>;WAPCre<sup>-</sup>) mice. In animals positive for the WAPCre transgene, the complete open reading frame of c-myc will be excised upon Cre expression (Figure 1(a)). To assess onset and extent of WAPCre expression, we performed immunohistochemistry (IHC) against Cre recombinase on sections from mutant mammary glands (Figure 1(b)). Cre expression was first detected at day 14.5 of pregnancy in scattered luminal alveolar cells. The number of Cre-expressing cells increased continuously until after parturition, when positive staining for Cre was seen in essentially all luminal cells. To monitor recombination, we performed polymerase chain reaction (PCR) on genomic DNA isolated from mammary glands at different developmental stages. The 220 base pair band, indicating the presence of the recombined allele, was first detected at day 14.5 of pregnancy (Figure 1(c)), consistent with the results from IHC. Starting then, levels of c-myc mRNA decreased rapidly in glands of mutant mothers and were essentially undetectable throughout lactation (Figure 1(d)). With the commercially available antibodies, it has not been possible to detect c-Myc in the lactating mammary gland by IHC (data not shown; Klinakis et al. [36]). Since the half-life of c-Myc protein and mRNA is short [37], it is likely that mutant glands have little or no c-Myc by the onset of lactation. Finally, mRNA levels of the cell cycle inhibitor p21<sup>Cip</sup>, a well-studied target of c-Myc-mediated repression [38,39], were upregulated in c-Myc-deficient glands during lactation (Figure 1(e)), which is in agreement with the functional loss of c-Myc in mutant glands.
Figure 1
Targeted disruption of c-Myc in the mammary gland. (a) Schematic diagram of c-myc floxed allele and recombined allele after Cre-mediated excision of floxed region. The position of the 220 base pair (bp) polymerase chain reaction (PCR) product for detecting recombined allele is indicated. (b) Immunohistochemistry against Cre (brown nuclei) on paraffin sections of mutant mammary glands. Representative staining from different stages of pregnancy (P), lactation (L), and involution (I). Scale bar, 100 μm. (c) PCR on genomic DNA from glands taken at the indicated days from wild type (WT) (W) and mutant (M) mice to detect the recombined c-myc allele (220 bp) indicated in (a). (d) Semi-quantitative reverse transcription-PCR showing c-myc and β-actin mRNA levels in glands of WT and mutant mice removed at two time points during a first pregnancy and at seven times in lactation. (e) Relative expression levels of p21\(^{CIP1}\) determined by qPCR in WT and mutant glands at four different time points in lactation. Results are the average of duplicate measurements with β-actin mRNA levels as reference.
c-Myc mutant mothers display a lactation defect with less efficient milk production

Monitoring survival and weight of newborn pups is routinely used as a measure of lactation [40]. Thus, we performed a pup weight analysis to examine the efficiency of milk production in WT and mutant females. Growth curves generated from seven foster pups per mother showed that pups nursed by mutant mothers grew significantly slower compared with pups nursed by a WT mother (Figure 2(a), left panel). However, when comparing a mutant mother nursing only two foster pups to a WT mother nursing six pups, there was no significant difference in pup body weight (Figure 2(a), right panel). This suggests that milk quantity, but not quality might be affected in c-Myc deficient glands.

To test this hypothesis, we first examined milk composition. Milk samples taken from WT and mutant mothers at day 14.5 and 15.5 of lactation were analyzed for protein, lactose and fat content, the three major milk components. On a Coomassie stained gel, milk protein pattern and concentration were identical in equal volumes of milk from WT and mutant mothers (Figure 2(b), casings are indicated) [see also Marte et al. [41]]. Furthermore, the concentration of lactose, the major carbohydrate and osmole in milk, as well as the fat content were determined in milk samples from a group of five animals made up of WT, heterozygous (showing no overt phenotype) and mutant mothers (Figure 2(c) and 2(d)). Lactose concentration was determined in a colorimetric assay on skim milk samples, whereas fat content was measured as the ratio of cream layer length over total milk length after centrifugation (creamocrit) (Lucas et al. [42]). While one heterozygous mother showed a slightly decreased lactose concentration, likely due to natural variation (Figure 2(c)), there were no consistent alterations in either lactose or fat content within the samples.

Next, to compare the approximate amount of milk produced in the lactating glands from WT and mutant mothers, the following experiment was performed. In the first setting, mothers were sacrificed immediately after removing them from their actively suckling pups. In the second setting, mothers were removed from their pups and sacrificed 2 hours later, which allows the glands to fill with milk. When comparing high magnifications of whole mount preparations taken from actively nursing mothers, glands from WT and mutant mice looked nearly identical (Figure 2(e), panels a and b). However, only the WT females showed clear signs of milk-filling, displaying large, distended alveoli after 2 hours without pups (Figure 2(e), panel c, arrows), while glands of mutant mothers appeared only slightly distended (Figure 2(e), panel d).

Finally, we examined the milk proteins via a Western analysis carried out on protein lysates made from lactating mammary glands of WT and mutant mothers. Equal amounts of protein were loaded and membranes probed with a rabbit anti-milk serum [41], producing a staining pattern of multiple milk proteins (Figure 2(f)). The blot shows that mutant protein lysates contain less milk protein than the corresponding WT lysate at day 5.5, 10.5, and 15.5 of lactation. The level of α-tubulin, used as a loading control, was the same in each paired WT and mutant sample. Taken together, these results clearly suggest that the reduced nursing ability in c-Myc mutant mothers is due to decreased or slower milk production, while milk composition is essentially the same in mutant and WT mothers.

Alterations in alveolar density and secretory activity

To analyze the lactation defect in more detail, we investigated the morphology of glands from lactating, actively nursing WT and mutant mothers via H&E. Cytokeratin (Ck) 18-stained cross-sections were scanned (Figure 3(a)), revealing that mutant glands contained more unstained stromal area than WT glands. To obtain quantitative results, the area covered by alveoli (including epithelium and lumen) was measured and the ratio of alveolar area over total organ area was calculated (Figure 3(b)), revealing that alveolar area in mutant glands was significantly decreased, on average by 30% (lactation day 3.5) and 20% (lactation day 10.5). Interestingly, the number of alveoli per organ area was not altered (Figure 3(c)), suggesting that in mutant glands there is a reduction in the size of alveoli, which could be due to smaller and/or fewer alveolar cells or less milk, even in an active nursing state.

To analyze this in more detail, proliferation and apoptosis were investigated in lactating glands. We did not detect any difference in BrdU incorporation between WT and mutant glands (data not shown), nor were shed cells apparent in the lumens (for example, when looking at high magnifications of Figures 1(b) and 3(a)), suggesting no dramatic alterations in cell number. Thus, we examined the glands via electron microscopy to look directly at the secretory activity of alveolar cells. The endoplasmic reticulum forms highly organized, parallel strands, from which secretory vesicles bud to fuse into the alveolar lumen (Figure 3(d)). When comparing day 7.5 lactating WT and mutant glands, mutant cells are dominated by parallel regions of thin regular endoplasmic reticulum. In contrast, WT cells contain more dilated reticulum and budding vesicles (arrows), indicating high protein synthesis activity. The result was confirmed in two pairs of day 4.5 lactating mice (not shown). The non-dilated endoplasmic reticulum in c-Myc mutant glands suggests a defect in protein synthesis, at the cellular level.

C-Myc controls biosynthetic activity in the mammary gland

The previous results suggest that c-Myc loss in alveolar cells might cause a general defect in milk production,
Figure 2
Ablation of c-Myc in mammary glands results in impaired lactation due to reduced milk volume. (a) Growth analysis of pups nursed by wild type (WT) or mutant mothers. Data are shown as average body weight plus standard deviation. Left panel: analysis of three littermate mothers nursing seven WT pups each, \( \bar{x} = 2.2 \times 10^{-5}, P = 1.1 \times 10^{-9} \). Right panel: comparison of a WT mother with six pups to a mutant mother nursing two pups (all pups WT littermates). NS = not significant, \( P = 0.52 \). (b) Milk protein composition in milk obtained from WT (W) or mutant (M) mice at lactation day 15.5. Freshly collected milk was diluted 1:20 in phosphate-buffered saline and 5 or 10 µl loaded on a 15% SDS-gel for Coomassie staining. (c) Measurement of lactose concentration in milk collected at lactation day 14.5. In a colorimetric assay, lactose concentration is determined as concentration of free galactose in lactose-treated skim milk. Results from five animals with the indicated genotype are shown as average value of duplicate measurements. (d) Analysis of fat content in the same milk samples used in (c), presented as percentage of fat layer length over total length of milk. Results are the mean ± standard deviation of three measurements per animal. (e) Whole mounts of WT and mutant mammary glands collected on lactation day 0.5. Mothers were sacrificed directly after removing them from pups (a, b), or after 2 hours without pups to allow filling of glands with milk (c, d). Arrows point to distended alveoli in WT gland. Scale bar, 500 µm. (f) Western analysis of milk proteins (loading: 1 µg per lane) and α-tubulin (loading: 9 µg per lane of the identical lysates) of WT and mutant mammary gland lysates at lactation day 5.5, 10.5, and 15.5. Blot was probed with anti-milk serum; two different exposure times are shown.
including milk components and the enzymes involved in their synthesis. This was analyzed in more detail, first by examining mRNA levels of milk proteins and enzymes that are strongly upregulated in lactating mammary glands [43,44]. Transcripts encoding α-lactalbumin (Lalba) and β-casein (Csn2), both milk proteins, the former also the rate-limiting co-factor for lactose synthesis [45], as well as Δ6 fatty acid desaturase 2 (Fads2), stearoyl-CoA desaturase 2 (Scd2), elongation of very long chain fatty acids (Elov11), and aldolase C (Aldo3), enzymes involved in lipid synthesis [44], were measured by semi-quantitative reverse transcription (RT)-PCR. All transcripts were expressed at comparable levels in WT and mutant glands analyzed between lactation day 2.5 and 10.5 (Figure 4(a)), including Fads2, Scd2 and Elov11 that are described Myc targets in other systems (http://www.myc-cancer-gene.org/index.asp, Zeller et al. [46]). This suggests that regulation of milk production by c-Myc might occur by a non-transcriptional mechanism.

Next we investigated mRNA translation in WT and mutant glands by performing polysome fractionation on mammary gland lysates obtained at lactation day 4.5. This technique allows the separation of mRNAs along a sucrose gradient depending on their ribosomal load. When overlaying profiles from WT and mutant glands according to their monosome peaks, a change in the average size of polysomes was evident in c-Myc deficient glands, with the peak being shifted to smaller polysomes (Figure 4(b), upper panel). Results from one pair of WT and mutant animals are shown; three additional pairs of animals were examined, yielding similar results (data not shown). As a control, we performed polysome fractionations on livers obtained from the females used for generating the mammary gland profiles. WT and mutant mice retain c-Myc in the liver since WAPiCre is not expressed there. The polysome distribution from livers of WT and mutant females was nearly identical (Figure 4(b), lower panel), showing that the altered polysome distribution is specific for c-Myc-deficient mammary glands. These results suggest that there is a general reduction in translation efficiency in mammary glands in the absence of c-Myc.

In addition to Pol II targets, c-Myc controls Pol I-mediated rRNA and Pol III-mediated tRNA and SS rRNA transcription, thereby regulating cellular physiology at multiple levels [1,47-49]. Accordingly, we analyzed a panel of Pol I, II and III c-Myc targets implicated in ribosome biogenesis and translation. The results from qPCR are displayed as relative expression levels in mutant mice, compared with matched WT littermates; the data are from two pairs of mice at the indicated times in lactation (Table 1). mRNAs encoding nucleolin and nucleophosmin, which are involved in ribosome biogenesis, mRNAs encoding large and small ribosomal subunit proteins, and the mRNA for poly(A)-binding protein 1 (PABPC1), involved in translation, all showed a decrease in samples from mutant females. In particular, the ribosomal protein encoding mRNAs were strongly affected, frequently being more than two-fold downregulated in c-Myc-deficient glands (Table 1, values below 0.50). Furthermore, the levels of SS rRNA as well as the rapidly processed 5′-external transcribed spacer of the 45S rRNA precursor [7], were generally lower in c-Myc mutant glands. This suggests that the decreased translation efficiency in c-Myc mutant glands is due to a general impairment of ribosome biogenesis and translation.

Finally, we examined the translational efficiency, that is, ribosomal load, of specific mRNAs using RNA isolated from each fraction of the polysome gradient. The mRNAs encoding Lalba, Csn2, Fads2, Scd2, Elov11 and Aldo3 each shifted to smaller polysomes, with the peaks in fractions 7 to 9 in mutant versus 8 to 10 in WT glands (Figure 4(c), upper panel, open arrow heads). Interestingly, while each of these transcripts is expressed to the same level in WT and mutant mammary glands (Figure 4(a)), this shift clearly shows that they are less efficiently translated. In contrast to the mRNAs encoding proteins directly involved in milk production, the mRNA distribution of β-actin, CK18 and GAPDH along the polysome gradients was essentially the same in WT and mutant glands (Figure 4(c), lower panel, open arrow heads). To confirm that the observed reduced translation efficiency results in less protein production in mutant glands, we performed a Western analysis for β-casein on mammary gland lysates (Figure 4(d)). Compared with the α-tubulin loading control, there is a clear reduction in casein levels in lysates of mutants compared with WT littermates. Taken together, these results show that a reduction in translation efficiency is likely to be responsible for slower milk production in c-Myc mutant glands.

Delayed proliferative response in c-Myc mutant mammary glands

c-Myc loss has an effect on cell cycle progression and proliferation in many organs [25,28,29,31,33]. Thus, we investigated if c-Myc loss affects proliferation during pregnancy. The WAPiCre model is particularly suited for studying proliferation in a second pregnancy since a population of WAPiCre expressing cells does not undergo a secretory fate, but survives lactation and involution. These cells are termed Pi-MECs (for parity-identified mammary epithelial cells) (see also Smith and Medina [50]) and function as progenitor cells for epithelium-forming alveolar structures during ensuing rounds of pregnancy and lactation [51,52]. In our model, cells in c-myc−/−;WAPiCre+ mice that survive involution will have lost c-Myc due to Cre expression during the first pregnancy. Consistent with these characteristics, the recombined c-myc allele was detected in non-pregnant, parous females, and in all stages of a second pregnancy (Figure 5(a)), in contrast to the first pregnancy where recombina-
Figure 3
Alterations in alveolar density and secretory activity. (a) Immunohistochemistry against cytokeratin (CK) 18 on sections of wild type (WT) and mutant glands to visualize epithelium (brown). For optimal comparison, a central part of each gland containing the lymph node was taken. Lymph nodes (blue) and stroma display no CK18 staining. (b) Quantification of alveolar area per total organ area (excluding lymph node) from sections processed as shown in (a). Two sections per animal were quantified and the average was used for calculations. Results are the mean ± standard deviation of four and three pairs of animals quantified on lactation day 3.5 and 10.5, respectively. *, P = 0.00057; **, P = 0.0043. (c) Number of alveoli (including mean ± standard deviation) measured in four pairs of animals at lactation day 3.5, which were used for area measurement in (b). Numbers were calculated per analyzed area, and values from WT glands were set to 1. NS = not significant, P = 0.25. (d) Electron microscopy pictures from day 7.5 lactating WT and mutant glands. Arrows point to regions of dilated, vesicle forming endoplasmic reticulum. Scale bar, 500 nm.
tion was first detectable at day 14.5 (Figure 1(c)). Furthermore, c-myc mRNA levels are very low during a second pregnancy and lactation in mutant, compared with WT glands (Figure 5(b)).

In the normal mammary gland c-myc mRNA is highest between day 6.5 and day 12.5 of pregnancy then drops to baseline for the remainder of pregnancy and throughout lactation [53]. In our model, during the first pregnancy Cre activity, hence c-Myc deletion is maximal early in lactation, a time when it has not been possible to detect c-Myc by IHC (data not shown; Klinakis et al. [36]). However, since the recombined c-myc allele was detected in all stages of a second pregnancy (Figure 5(a)) and c-myc mRNA levels are very low in mutant glands (Figure 5(b)), we performed IHC staining for c-myc on sections prepared from second pregnancy day 6.5 mammary glands. c-Myc staining was evident in sections prepared from WT females (see Additional file 1), although not as strong as the day 10.5 embryonic liver positive control [22]. In contrast, in the mutant glands, c-Myc staining was absent in most of the epithelial clusters. These results clearly show that in c-myc<sup>−/−</sup>;WAP<sup>Cre</sup> mice c-Myc mRNA and protein are lost.

To monitor proliferation during pregnancy, IHC for Ki-67, which stains all but G0 cells, was performed (Figure 5(c), left). Furthermore, cyclin D1, which is preferentially expressed in the mammary gland and is essential for proliferation [54] was analyzed by IHC (Figure 5(c), right). In sections from WT glands the majority of cells were actively cycling at pregnancy day 6.5, displaying positive Ki-67 staining, as well as high levels of cyclin D1. In striking contrast, in mutant glands analyzed on the same day, the majority of cells were Ki-67 negative, and had low or undetectable cyclin D1, showing that most cells were not proliferating. By pregnancy day 14.5, however, the majority of mutant cells were cycling, showing that the slower proliferative response was surmountable. Mutant glands at day 14.5 resembled WT glands at day 6.5, whereas by day 14.5, WT glands displayed advanced development with many lumens-forming, alveolar clusters. Of note, levels of N-myc and L-myc were the same as in WT glands, showing that there was no compensation at the mRNA level in glands lacking c-Myc (Figure 5(d)). In summary, the results indicate that in the absence of c-Myc, alveolar cells show a delayed proliferative response at the start of pregnancy.

**Delayed, but successful differentiation in c-Myc mutant glands**

The slower proliferation in the mutant glands resulted in delayed differentiation, which was monitored by IHC against milk proteins (Figure 6(a)). While the WT gland at pregnancy day 14.5 was producing milk, as shown by the milk-filled lumen of the alveoli, the small alveolar clusters in the mutant gland were essentially empty and no milk was detected. By pregnancy day 16.5, however, alveoli occasionally contained cytoplasmic lipid droplets (Figure 6(a), red circles, insert), indicating that the mutant cells had begun differentiation and milk production. Indeed, despite the delay in development, milk production was successful since mutant mothers were able to nurse their pups after parturition, albeit with pups showing reduced body weight (data not shown). Interestingly, expression of the WAP<sup>Cre</sup> transgene was also delayed in a second pregnancy (Figure 6(b)). A control female (c-Myc<sup>−/−</sup>;WAP<sup>Cre</sup>) in its second pregnancy and lactation showed scattered Cre staining at pregnancy day 16.5 and ubiquitous staining at lactation day 3.5 (as in Figure 1(b)). In contrast, mutant glands displayed almost no Cre-expressing cells at day 16.5 of a second pregnancy and only scattered positive cells at lactation day 3.5. Importantly, at day 10.5 of the second lactation, the mutant glands showed ubiquitous Cre expression, indicating that the transgene had not been silenced (Figure 6(b)). These results suggest that in the c-Myc mutant glands the WAP<sup>Cre</sup> transgene and endogenous milk protein genes show a similar delay in their expression pattern, very likely reflecting the slower proliferative response.

Finally, quantification of the alveolar density showed that during a second round of pregnancy and lactation, c-Myc mutant glands displayed a strongly reduced alveolar area (Figure 6(c)). With more than a 40% reduction on lactation day 3.5, this effect is more severe than the 30% decrease observed in a first pregnancy (Figure 3(b)). The reduced alveolar area is also evident in whole mount preparations from WT and mutant females obtained at the same time points (Figure 6(d)). The results might be explained, in part, by the slower proliferation leading to an incomplete alveolar expansion in the mutant glands (Figure 5(c)). In conclusion, the data suggest that c-Myc is dispensable for secretory differentiation, however, due to slower proliferation there is also a delay in differentiation in c-Myc mutant mammary glands.

**Effects on progenitor cells in c-Myc mutant glands**

Considering the more severe phenotype in the second pregnancy, we performed additional experiments to investigate the role of c-Myc in mammary progenitor cells. A quantification of the alveolar number from three pairs of WT and mutant females, analyzed at lactation day 3.5 of a second pregnancy, revealed a significant reduction in c-Myc mutant glands (Figure 7(a)), suggesting that mutant glands start the second pregnancy with fewer alveolar progenitor cells. Importantly, there was no difference in the alveolar number between WT and mutant glands measured at the first lactation (Figure 3(c)).

To functionally investigate mammary progenitor cells, we performed reconstitution experiments into cleared mam-
Figure 4
Altered translation efficiency in mutant mammary glands. (a) Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) on α-lactalbumin (Lalba), β-casein (Csn2), Δ6 fatty acid desaturase 2 (Fads2), stearoyl-CoA desaturase 2 (Scd2), elongation of very long chain fatty acids (Elolv1), aldolase C (Aldo3) and β-actin (Actb) in wild type (WT) (W) and mutant (M) glands taken at different time points during lactation. (b) Polysome profiles of day 4.5 lactating mammary glands (upper panel) and corresponding livers (lower panel) of the same animals. Profiles were overlaid according to their 80S peak. Hollow arrowheads mark the peaks of the polysomal fractions. (c) Semi-quantitative RT-PCR on mRNA isolated from the indicated gradient fractions from WT and mutant polyisomes. mRNA distribution analyzed for genes described in (a) (upper panel) and ‘control genes’ β-actin, CK18, and GAPDH (lower panel). Hollow arrowheads indicate the peak of each mRNA distribution along the gradient. (d) Western analysis of β-casein (loading: 0.25 μg per lane) and α-tubulin (loading: 50 μg per lane of the identical lysates) on WT and mutant mammary gland lysates obtained at three time points in lactation.
mary fat pads. Pieces of mammary glands from WT and mutant mothers were transplanted into NOD/SCID recipients. Donor glands were taken from lactation day 5.5, a time point where CRE activity is maximal and most cells will have lost c-Myc. Recipients were sacrificed after 8 weeks in order to examine survival and outgrowth potential of mammary progenitor cells. The results from two independent experiments are summarized in Figure 7(b). Epithelium from WT donors reconstituted a ductal network in all recipients. A representative outgrowth that filled around 30% of the gland (+ + ) is shown in Figure 7(c). In contrast, in 60% of the cases, transplanted epithelium from mutant donors failed to grow out and only rudimentary ductal trees were detected in the recipients (Figure 7(c), mutant, left panel). In the cases when mutant epithelium formed ductal outgrowths (Figure 7(c), mutant, right panel), these were similar to those formed by WT epithelium. A PCR analysis showed that the recombined allele could be detected in DNA recovered from two positive (+ + ) mutant outgrowths (Figure 7(d)), showing that c-Myc-deficient epithelial cells survived and likely contributed to outgrowth formation. In conclusion this suggests that c-Myc has an impact on mammary gland progenitor cell survival and/or proliferation.

Discussion
Since the early 1980s, numerous investigations focused on c-Myc, exploring its role in normal organ physiology, as well as in tumor biology (for recent reviews see Eilers and Eisenman [11], Meyer and Penn [55]). Results from mammary gland transgenic models implicate c-Myc with lineage commitment during embryonic development [56], with precocious proliferation and differentiation during pregnancy [57], and with premature involution [58]. c-Myc has also been intensely studied in breast cancer [16,59,60], and in mouse models of mammary cancer [17-19]. Here we present for the first time physiological functions of c-Myc during mammary gland development using a conditional knock-out approach. Given the ability of c-Myc to regulate transcription of a large number of genes, thereby impacting on all aspects of cellular physiology, it is not surprising that loss of c-Myc in the mammary gland affects different processes. We observed strong phenotypes at the start of pregnancy and during lactation; whereas during involution no alterations in the c-Myc mutant glands were observed (data not shown). At the

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*aResults are relative expression levels in mutant mice compared with levels in the corresponding wild type (WT) littersmates. Four mutant mice (Mu-1 to -4) were analyzed on lactation day 2.5 and 5.5 and each value is relative to the matched WT value. bDirect targets of transcription activated by c-Myc, chosen from http://www.myc-cancer-gene.org/index.asp [46].
start of pregnancy, c-Myc-deficient alveolar cells were impeded in their proliferative response, resulting in a delayed ability to differentiate. Moreover, mutant glands displayed slower milk production, a general decrease in translation and reduced expression levels of ribosomal RNA and proteins. Finally, the mutant mammary epithelium had a reduced ability to grow when transplanted into mammary fat pads suggesting that c-Myc has a role in progenitor cell proliferation and/or survival.

Role of c-Myc in proliferation at pregnancy

Pregnancy is a time of intense cell division, and c-Myc levels increase early in this developmental phase [our observations; Master et al., [53]]. Indeed, cells from WT females are essentially all cycling, showing high levels of cyclin D1 early in pregnancy. In contrast, c-Myc-deficient alveolar cells remained in G0, displaying lower levels of cyclin D1, and were delayed by at least 6 days in their proliferation. The extensive alveolar development occurring during the first half of pregnancy is dominated by progesterone (for
reviews see Naidu et al. [61], Neville et al. [62]) and c-Myc might have a role in mediating the response to this steroid hormone. In breast cancer models, progesterone induces c-Myc expression [63] via a progesterone receptor regulatory element upstream of c-myc [64]. c-Myc has a well-described role in cell growth and proliferation [1]. Thus, one mechanism underlying the slower proliferation might be related to the established role of c-Myc in controlling expression of cell cycle regulators [65], and progesterone might be the upstream regulator of c-Myc. Moreover, c-Myc effects on proliferation might be more indirect by regulating production of paracrine factors, many of which have been shown to be required during alveolar development (see, for example, Naidu et al. [61]). It should also be mentioned that since this phenotype was observed in the second pregnancy, it might result from a
secondary effect of reduced translation and biosynthetic activity during preceding developmental stages. While c-Myc has been described to couple cell growth to cell division [66], the question whether the observed effect in the mammary gland is secondary or intrinsic to c-Myc loss, can be better addressed with alternative Cre models.

During the second half of pregnancy, c-Myc-deficient cells were proliferating and the mutant gland did 'catch up' with the WT, as attested to by the ability of mutant mothers to nurse. That this alveolar development is due to c-Myc-proficient 'escaper' cells is very unlikely, since at pregnancy day 14.5 c-Myc levels are still very low while Cre expression and recombination only re-starts at day 16.5. An obvious reason explaining this phenotype might be a slow compensation for c-Myc loss by other Myc family members, since they are, in part, functionally redundant to c-Myc [22,33]. While this cannot be ruled out, there was no observable increase in L-myc or N-myc expression at day 14.5 of pregnancy, a time point when mutant cells were dividing. While we can only speculate, it is possible that this developmental stage proceeds independently of c-Myc. Indeed, the second half of pregnancy is controlled by ligands activating prolactin receptor signaling [62], and the Elf5 transcription factor was shown to be a key mediator of prolactin receptor signaling in promoting alveolar development [67]. Thus, we propose a model whereby c-Myc is required early in pregnancy, potentially down-

![Graph](image1)

**Figure 7**

**Effects on mammary progenitor cells.** (a) Number of alveoli (including mean ± standard deviation) measured in three pairs of animals at second lactation day 3.5, which were used for area measurement in Figure 6(c). Numbers were calculated per analyzed area, and values from wild type (WT) glands were set as 1. *P = 0.018. (b) Summary of two transplantation experiments with a total of 10 NOD/SCID mice grafted contraterilaterally with WT and mutant epithelium. Positive outgrowths were classified as + (filling <25% of cleared gland), ++ (filling 25% to 50%) or +++ (filling about 75%). (c) Wild type: whole mount of virgin recipient gland containing a ++++ outgrowth from transplanted WT epithelium, 8 weeks after transplantation. Mutants: whole mounts of a negative and a positive (+++ ) outgrowth obtained from mutant epithelium (left and right panel, respectively). Scale bar, 1 mm. (d) Polymerase chain reaction for the recombined allele as described in Figures 1(c) and 5(a), performed on DNA isolated from two recipient mice bearing WT (W) and positive (+++) mutant (M) outgrowths. Control: lactating mutant control; R-1, R-2: NOD/SCID recipient 1 and 2.
stream of progesterone signaling, but is dispensable for alveologenesis during the second half of pregnancy.

**Role of c-Myc in translation during lactation**

We also studied the role of c-Myc during lactation, when the gland devotes its energy to the coordinately regulated process of milk production. mRNAs encoding various milk proteins and enzymes that are strongly upregulated during lactation [43,44] were found at similar levels in WT and mutant mammary glands, suggesting that loss of c-Myc does not impair their transcription. Furthermore, milk produced by the mutant glands is identical in composition to that made in control glands and pups were healthy, albeit with a slower weight gain when nursing on mutant mothers. This phenotype, suggesting that there was a slower rate of milk production in the mutant glands was investigated and shown to result from a general decrease in translation efficiency.

Numerous studies have described c-Myc’s multifaceted roles in mRNA translation, either via transcriptional [48] or non-transcriptional mechanisms [13]. Our results show that c-Myc controls transcription of various target genes with important roles in translation. The c-Myc mutant glands displayed lower levels of mRNA encoding PABPC1, which is involved in translation initiation, and mRNAs encoding nucleophosmin and nucleolin, both involved in ribosome biogenesis. Interestingly, c-Myc loss in intestinal crypts also led to reduced biosynthetic activity, characterized by a loss of nucleolar organizing regions and decreased expression of nucleophosmin [31]. Moreover, very compelling results showing c-Myc’s importance during lactation arose from our examination of ribosomal RNAs, and mRNAs encoding ribosomal proteins; these showed a marked reduction in c-Myc mutant glands. Thus, c-Myc is needed for efficient Pol I, II and III transcription in the mammary gland. Since ribosome availability is the rate-limiting step in protein synthesis [68], the strong decrease in the RNA level of components needed for ribosome biogenesis, combined with others not important for translation, very likely explains the reduced milk production in the c-Myc mutant glands. Although we did not examine cell size in the mammary gland, it is possible that the reduced biosynthetic activity could also result in smaller cells, as c-Myc regulates cell size in some models/organisms analyzed [25,26,31,69]. It will be interesting to address this aspect in detail in future studies.

Hormonal induction of milk production during lactation is subject to transcriptional and translational control mechanisms (reviewed in Rhoads and Grudzien-Nogalska [70], Rosen et al. [71]); the latter have been extensively studied using polysome fractionation techniques [72,73]. Here we show that c-Myc has a general role in translation efficiency, as attested to by the reduction in the average size of the polysomes in its absence; however, some selectivity was also uncovered. While mRNA transcripts of milk proteins (LaIba and Gsn2) and enzymes important for milk production (Pads2, Scd2, Elov1, and Aldo3) were shifted to smaller polysomes in c-Myc mutant glands, other mRNAs were not affected. Indeed, there was little or no change in the ribosomal loading of β-actin, CK18, and GAPDH mRNAs in the absence of c-Myc, suggesting that their translation efficiency is not altered. It is well established that different categories of mRNAs, referred to as ‘weak’ and ‘strong’, have diverse responses to general changes in translation [74] and mRNAs for house-keeping proteins are the least affected by external stimuli. Thus, one plausible mechanism underlying the translational selectivity could be that during lactation, a time when the energy of the organ is devoted to milk production, targets upregulated during the differentiation program would be most affected by the limited availability of ribosomes, while other mRNAs continue to be translated with constant ribosome occupation. Finally, a recent study showed that c-Myc stimulates translation by enhancing mRNA cap methylation and subsequent ribosomal loading [13]. This mechanism might also contribute to the selectivity that we uncovered in the mammary gland. In conclusion, we show here that c-Myc-deficient glands have reduced levels of ribosomal proteins and RNA, as well as proteins involved in ribosome biogenesis and translation. Therefore, by acting on many different components of the translation machinery, c-Myc has an important role in successful and efficient translation during lactation.

**Role of c-Myc in progenitor cell proliferation/survival**

The role of c-Myc in stem and progenitor cells has been intensively studied in different mouse models. In the bone marrow, two reports showed that loss of c-Myc leads to an accumulation of hematopoietic stem cells and a severe loss of the committed lineages due to impaired differentiation [32,34]. Moreover, elimination of both c-Myc and N-Myc in hematopoietic stem cells impairs self-renewal and leads to rapid apoptosis of stem cells [33]. In the skin, depletion of the epidermal stem cell population, due to insufficient amplification of the cells was observed following c-Myc deletion [25]. A function for c-Myc in mammary stem or progenitor cells seems likely, as the Wnt and Notch signaling pathways are believed to play important roles in mammary stem cells [75], and both can directly stimulate c-Myc expression [76].

Indeed, our results suggest that c-Myc plays a role in a subset of mammary gland progenitor cells, the PI-MECs. This population of WAPCre expressing cells does not undergo a secretory fate, but survives lactation and involution [51,52]. A model of the mammary stem cell hierarchy suggests that the PI-MECs are contained in the Sca-1 negative population and function as alveolar progenitors during...
pregnancy [77]. As c-Myc mutant glands show a reduced number of alveoli in the second pregnancy/lactation, it is likely that fewer progenitor cells were present following c-Myc loss in the first pregnancy and lactation. This hypothesis is further supported by results from the transplantation experiments. The reduced outgrowth capacity found with the mutant epithelium suggests that alveolar progenitors have an impaired ability to proliferate or to survive in the absence of c-Myc.

Relevance of c-Myc in cancer
The importance of c-Myc in cancer was established more than 20 years ago and much effort has gone into studying all aspects of oncogenic c-Myc (reviewed in Meyer and Penn [55]). c-Myc is aberrantly expressed in most breast cancers as a result of gene amplification or from alterations in signaling pathways that impact on c-Myc RNA or protein levels. Myc and many of its target genes were recently shown to be strongly expressed in basal, ERα negative breast tumors, allowing them to proliferate in the absence of estradiol-induced signaling [78]. Our studies on c-Myc in normal development have important implications for breast cancer. Indeed, it was shown in a c-Myc-induced tumor model that Myc's ability to induce protein synthesis was a major factor contributing to aberrant growth and genic lesions [79].

Despite the phenotypes in pregnancy and lactation, the effects of c-Myc loss in the mammary gland are generally well tolerated, which is of interest considering c-Myc as a target in cancer therapy [14,80]. Recent studies have evaluated the role of c-Myc in tumor onset and maintenance and have also addressed the side effects of Myc-targeting. In the intestines, where inactivation of adenomatous polyposis coli (APC) is a key event in colorectal cancer development, c-Myc is frequently overexpressed as a downstream β-catenin/T cell factor target. Interfering with c-Myc levels in mouse models with APC mutations rescued the observed phenotypes, leading to a reduction in tumor burden and increased survival [81,82]. Furthermore, by employing an inducible, dimerization-interfering Myc construct in a Ras-induced lung adenocarcinoma model, it was shown that Myc inhibition impaired tumor maintenance. Importantly, the 'side effects' observed in other organs disappeared rapidly after cessation of Myc inhibition [83]. Finally in Notch1-induced mammary tumors it was shown that ablation of c-Myc reduces tumor incidence and increases tumor latency, suggesting that Myc might be an attractive target in cancers with deregulated Notch signaling [36]. Multiple signal transduction pathways activate c-Myc [84], many of which are deregulated in breast cancer [85-87]. Future studies using different mammary tumor models will provide more insight into the role of c-Myc in tumor development and maintenance, and in its potential as a breast cancer target.

Conclusion
Our data revealed three interesting new roles for c-Myc in the mouse mammary gland. At the start of pregnancy, c-Myc loss resulted in delayed proliferative response and differentiation. During lactation, mutant glands showed reduced milk production and slower pup weight gain. Furthermore, c-Myc-deficient glands were generally impaired in translation efficiency and displayed reduced levels of ribosomal RNA and proteins. Finally, the results from transplantation assays suggest that c-Myc has a role in progenitor cell proliferation and/or survival. Our results provide new insight into Myc's physiological role in breast development, which might gain special importance considering c-Myc as a novel target in the aggressive basal breast cancer subtype.

Methods
Mouse strains

\( c\text{-mye}^{\text{38}} \) mice were mated with mice containing a single copy of the WAPCre transgene and pups were further intercrossed. Littermates with the genotype \( c\text{-mye}^{\text{38}/}\text{WAPCre}^{\text{WT}} \) or \( c\text{-mye}^{\text{38}/}\text{WAPCre}^{\text{HET}} \) (referred to as WT), \( c\text{-mye}^{\text{38}/}\text{WAPCre}^{\text{HET}} \) (mutant) and \( c\text{-mye}^{\text{38}/}\text{WAPCre}^{\text{HET}} \) (heterozygous) were used for all studies. Mothers were maintained with litters of six pups and only inguinal glands were taken in the experiments. For growth analysis, newborn pups were mixed and two to seven pups were placed with WT and mutant mothers. Body weight of each pup was measured regularly and the results presented as average weight ± standard deviation. For milk volume experiments, mothers were either directly sacrificed or after a 2 hour period without pups, to allow milk filling of the gland. For milking females, pups were removed from mothers for at least 4 hours, then mice were anesthetized with Ketavet (100 μl/10 g body weight intraperitoneal) and milk release was induced by intraperitoneal injection of 0.3 IU oxytocin. Milk was removed by applying gentle pressure and directly drawing it into capillary tubes or pipettes for further analysis. All animal experiments were carried out under the Swiss guidelines for animal safety.

DNA/RNA isolation, PCR and RT-PCR

Pieces of mammary glands were flash frozen in liquid nitrogen for RNA or DNA isolation. DNA was precipitated with ethanol after proteinase K digestion (56°C overnight). For detection of the recombinant c-mye allele, the following primers were used in a PCR: fwarz: 5'-AAATGCTGATCCTACTAAATTTACGGCTG-3'; rzw: 5'-TACAGCCC-AAAACCCACCAAG-3'. RNA was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription was carried out using the Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Buckinghamshire, UK) with oligo(dT)15 or random hexamer primers (both Promega, Madison, WI, USA) for mRNA or RNA detection, respectively. We used 2 μl and/or 4 μl cDNA for semi-quantitativa-
Results: Research article

tive PCR analysis. Detailed information (including primers for semi- and quantitative PCR) can be found in Additional file 2.

**Milk analysis**
Aliquots of milk were centrifuged in capillary tubes (30 minutes, 3,500 rpm) to determine the fat content (cream, atocrit), measured as the ratio of the upper cream layer length over total milk length [42]. Milk protein composition was analyzed by diluting fresh milk 1:20 in phosphate-buffered saline (PBS) and loading 5 µl and 10 µl on 15% SDS-PAGE, which was stained with Coomassie Blue. For measuring lactose content, samples of milk, frozen in liquid nitrogen and stored at -80°C, were thawed and centrifuged (20 minutes, 4°C, 3,000 g) and 10 μl of the lower aqueous phase were used in a colorimetric galactose/lactose assay-kit (BioVision, Mountain View, CA, USA).

**Immunohistochemistry**
For histological examination, the central region of inguinal mammary glands containing the lymph node was used. Glands were fixed in freshly prepared 4% paraformaldehyde in PBS and stored in 70% ethanol until embedding in paraffin. IHC was performed on 4 µm paraffin sections using the following antibodies: Cte [35], CK18 (Progen Biotechnik, Heidelberg, Germany), c-Myc (Upstate Biotechnology, 06-340, Lab Placid, NY, USA), Ki-67 (Lab Vision, Fremont, CA, USA), cyclin D1 (Cell Marque, Rocklin, CA, USA) and rabbit anti-milk serum [41]. Stainings were carried out with the Discovery XT Staining Module (Ventana Medical Systems SA, Strasbourg, France). Images were acquired with a Leica DFC420 camera on a Nikon Eclipse E600 microscope using Plan Fluar 10×/0.3, 20×/0.5, and 40×/0.75 lenses. When necessary, optimization of brightness and contrast was performed by standard procedures in Corel DRAW 13 and always applied equally to the whole set of images.

**Mammary gland mounts and electron microscopy**
For whole mount staining, inguinal glands were spread on glass slides, fixed overnight at 4°C in Tellyesnitzky's fixative and stained with iron-hematoxylin as described http://www.bcm.edu/roenlab. Images were captured by a Leica DFC420 camera on a Nikon Eclipse E600 microscope with a Plan Apo 4×/0.2 lens in the milk-filling experiments. Pictures of other whole mounts and transplants were taken on a Leica Z6 APO microscope with a Plan Apo 2.0× lens and a Leica DFC480 camera.

For electron microscopy, pieces of mammary gland were fixed in Karnowsky's fixative (3% paraformaldehyde, 0.5% glutaraldehyde in 10 mM PBS pH 7.4), washed, and post-fixed in 1% OsO₄. After dehydration with graded series of ethanol, samples were embedded in Epon and sections of 60 to 70 nm thickness were cut. Sections were double stained with uranyl acetate and lead acetate [88] and viewed in a FEI Morgagni 268D transmission electron microscope.

**Image analysis and statistics**
Images were taken with a Mirax Slidescanner (Zeiss AG, Zurich, Switzerland) using a 20×/0.5 lens (0.2 μm/pixel) and converted into standard TIFF format. Manual counting of alveoli and measurement of alveolar areas were performed on TIFF-files using the measurement module of ImageAccess (Imagic AG, Glattbrugg, Switzerland). For automatic detection and measurement of alveolar area versus total organ area, images taken with the same slidescanner were analyzed using Definiens Software (Definiens AG, Munich, Germany).

Statistical analysis for alveolar area quantification and pup weight analysis was performed with one-sided Student's t-test. For alveolar counts, the ratios of the numbers obtained from mutant versus corresponding WT littermate were tested for significant deviation from one using ‘one sample’ t-test.

**Lysate preparation and Western blot analysis**
Frozen pieces of mammary gland were ground to powder in liquid nitrogen and homogenized in RIPA buffer (50 mM Tris pH 8, 1% NP40, 0.5% sodium deoxycholate, 20% SDS, 150 mM NaCl) complemented with 5 mM ethylene glycol tetraacetic acid, 1 mM dithiothreitol (DTT), 20 mM sodium pyrophosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 mM phenylmethylsulfonylfluoride (PMSF) and 1 mM sodium orthovanadate. Lysates were subjected to SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). After blocking, membranes were incubated overnight at 4°C with primary antibodies against α-tubulin (Neomarkers, Fremont, CA, USA) and β-casein [89], or 1 hour at RT with rabbit anti-milk serum [41]. Signals were detected by using horseradish peroxidase-linked secondary antibodies (GE Healthcare) and enhanced chemiluminescent detection reagent (GE Healthcare).

**Polysome fractionation of mammary glands and livers**
Half of an inguinal mammary gland and a piece of liver (both approximately 80 to 120 mg) were flash frozen in liquid nitrogen. For extract preparation, tissue was ground to a white homogeneous powder in liquid nitrogen with 1 ml polysome buffer (10 mM Tris pH8, 150 mM NaCl, 5 mM MgCl₂, 1% NP-40, 1% DOC, 10 mM DTT, 50 μg/ml cycloheximide, 0.4 U/μl RNAsin, 1 mM PMSF, 20 μg/ml aprotinin and leupeptin, supplemented with complete protease inhibitor (Roche Diagnostics, Indianapolis, IN, USA)). After thawing, cell debris were removed by centrifugation (12,000 g, 10 minutes, 4°C) and 700 μl of supernatant were loaded on to a linear sucrose gradient (15% to 60% sucrose (w/v), in 10 mM Tris pH7.5, 140 mM NaCl, 1.5 mM MgCl₂, 10 mM DTT, 100 μg/ml cycloheximide). Gradients were cen-
trifuged in a SW41 Ti rotor (Beckman Coulter Inc., Fullerton, CA, USA) for 2 hours at 38,000 rpm at 4°C with brakes off. Twelve fractions of 0.5 ml were collected as previously described [90]. RNA was isolated using TriZol reagent as described above: 1 μl glycogen (20 mg/ml) was added to facilitate isopropanol precipitation of RNA.

**Transplantation of mammary epithelium into cleared fat pads of NOD/SCID recipients**

Inguinal mammary glands of 3- to 4-week-old NOD/SCID mice (body weight below 13 g) were cleared of endogenous mammary epithelium as described [91]. Donor epithelium was derived from mammary glands of day 5.5 lactating WT and mutant mothers and chopped into approximately 1 mm³ pieces. Outgrowth efficiency was monitored 8 weeks after transplantation by sacrificing non-pregnant recipients and staining mammary gland whole mounts as described above. Transplants were scored as successful when originating from a central part of the cleared gland with ducts growing in all directions [92]. Positive outgrowths were rated as + (filling <25% of the gland), ++ (filling 25% to 50%) and +++ (filling about 75%).

**Abbreviations**

APC: adenomatous polyposis coli; CK: cytokeratin; DTT: dithiothreitol; HIC: immunohistochemistry; PBS: phosphate-buffered saline; PCR: polymerase chain reaction; PMSF: phenylmethanesulfonylfluoride; RT-PCR: reverse transcription-polymerase chain reaction; WT: wild type.

**Authors’ contributions**

TS participated in the design of the study, carried out all of the experimental work and analyzed the data. PS designed and developed the image analysis strategy. AT participated in designing the study and provided the c-myc∆∆ mice. NEH conceived the study and participated in its design and coordination. TS and NEH wrote the manuscript. All authors read and approved the final manuscript.

**Additional material**

**Additional file 1**

Additional figure. Immunohistochemistry for c-Myc. (a) Fetal liver of a day 10.5 embryo as positive control [22] showing strong nuclear staining in dark violet (counterstain pink). Scale bar, 50 μm. (b) Wild type (WT) and mutant mammary glands at second pregnancy day 0.5, when c-Myc expression is highest. WT epithelium shows clear dark staining compared with mutant glands, shown in the upper panel in violet (with pink counterstain) and in the lower panel in red (no counterstain). Note that in the mutant gland (lower panel) some epithelial clusters retained c-Myc (insert b, red staining) while all other clusters are clearly Myc-deficient (insert a, no staining). Scale bars, 50 μm.

Click here for file

[http://www.biomedcentral.com/content/supplementary/1741-7007-7-63-S1.PDF]

**Additional file 2**

Supplementary methods. Condition for quantitative polymerase chain reaction (PCR) and all primers used in semi-quantitative and quantitative PCR analyses. Click here for file

[http://www.biomedcentral.com/content/supplementary/1741-7007-7-63-S2.PDF]

**Acknowledgements**

We are very grateful to X Ding (Friedrich Miescher Institute for Biomedical Research (FMI)) for excellent help with the polysome fractionation. S Schuepbach-Philipp (Ecole Polytechnique Fédérale de Lausanne (EPFL)) for teaching TS the transplantation method. N Dubois (EPFL) for many helpful suggestions on Myc-HIC. Y Oliveri and U Sauder (Biocenter, University of Basel) for the electron microscopy work, the Benedites-Alj laboratory (FMI) for providing the NOD/SCID mice and many helpful ideas, H Grosshans (FMI) and C Briskin (Swiss Institute for Experimental Cancer Research, Lausanne) for helpful discussions and S Bicher, A Ponti and M Studer (FMI) for expert technical support in histology, image analysis and statistics, respectively. We thank M Benedites-Alj and W Filipowicz (FMI) for suggestions on the manuscript. We are grateful to N Li, R Masson (former Hynes Laboratory members) and all members of the Hynes Laboratory for their support. This work was supported by the Novartis Research Foundation and in part by grants to AT from the Swiss National Science Foundation, the EU FP6 Program ‘INTACT’ and the EU FP7 Program ‘EuroSysTem’.

**References**


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Additional files

Additional file 1

File format: PDF

Title: Additional figure

Description: Immunohistochemistry for c-Myc. (A) Fetal liver of a day 10.5 embryo as positive control [22] showing strong nuclear staining in dark violet (counterstain pink). Scale bar, 50 µm. (B) Wild type (WT) and mutant mammary glands at second pregnancy day 6.5, when c-Myc expression is highest. WT epithelium shows clear dark staining compared with mutant glands, shown in the upper panel in violet (with pink counterstain) and in the lower panel in red (no counterstain). Note that in the mutant gland (lower panel) some epithelial clusters retained c-Myc (insert b, red staining) while all other clusters are clearly Myc-deficient (insert a, no staining). Scale bars, 50 µm.
**Additional Figure:** IHC for c-Myc

**A**
Embryonic liver (day 10.5)

**B**
Wild type  Mutant

PV(16.5)
Additional file 2

File format: PDF

Title: Supplementary methods

Description: Condition for quantitative polymerase chain reaction (PCR) and all primers used in semi-quantitative and quantitative PCR analyses.
Stoelzle et al.  **Supplementary Methods**

**Semi-quantitative and qPCR**
Quantitative qPCR was performed using the Absolute SYBR Green ROX Mix (Thermo Scientific, Waltham, MA) and 1 µl of cDNA. Relative expression was determined in a ABI Prism 7000 cycler (Applied Biosystems Inc., Foster city, CA) in duplicate measurements, using $\beta$-actin levels as reference. All qPCR reactions were performed with the following cycling parameters: 50°C 2 min, 95°C 15 min, 40 cycles of 95°C 15 sec, 60°C 20 sec, 72°C 1 min.

The following specific primers were used for semi-quantitative or quantitative PCR:

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
</tr>
</thead>
</table>
| c-Mye  | fw: 5'-GTAATTCAGCAAGACAGAGAAGG-3'  
|        | rw: 5'-TGGGTGAAGTCTACGTTGAG-3'     |
| $\beta$-actin | fw: 5'-TGGGATCCACAGAGGATTCCATA-3'  
|        | rw: 5'-GATGGAACAGGATTCCATA-3'      |
| p21Cip1 | fw: 5'-GCCAAGTGCTGGCCTGTC-3'       
|        | rw: 5'-AGACCAATCTGCGCTTGG-3'        |
| Lactba | fw: 5'-CCATTAAGACATAGATGGCTATC-3'   
|        | rw: 5'-CAGGTAGCTTTTCAGAGCAC-3'      |
| Csn2   | fw: 5'-ACTACATTATGATCCCTCTCGAC-3'   
|        | rw: 5'-TGCTACTTTGCTGCAGAAATACAG-3'  |
| Fads2  | fw: 5'-TCTTCTGGCTTGCAGACT-3'       
|        | rw: 5'-TCTTCTGGCTCAGACT-3'          |
| Scd2   | fw: 5'-ACAACCTACCACCGTCTTC-3'       
|        | rw: 5'-GCTTCTGGGACAGGAACAG-3'       |
| Elov1l | fw: 5'-CCTAAGTGCTCAGGACTGC-3'      
|        | rw: 5'-CAGCCCTGAGTCTCTTCC-3'        |
| Aldo3  | fw: 5'-AAGGAGGAGCCCTAAGCTC-3'      
<p>|        | rw: 5'-CCGACAAACCTCTTCTGC-3'        |</p>
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<th>Reverse Primer (5'→3')</th>
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<td>5'-GCCTTGTGATGTTGTTGTCATC-3'</td>
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<tr>
<td>GAPDH</td>
<td>5'-TTCAATGGACCTCAACTATCAG-3'</td>
<td>5'-GTGCCAGTGATGGCATGGAC-3'</td>
</tr>
<tr>
<td>Nucleophosmin</td>
<td>5'-TCCTGGAGGTTG-GAACAAGG-3'</td>
<td>5'-ACCCTTGTACCTCGTGTTG-3'</td>
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<td>5'-TTGATCGTGCTCCAGAGTCG-3'</td>
<td>5'-TGAGGGTAGCAACCTCCC-3'</td>
</tr>
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<td>RPL3(^a)</td>
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<td>5'-ATCTCATCTGCCCACAC-3'</td>
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<tr>
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<td>5'-CGATCCTGTCAGGTCTCTC-3'</td>
</tr>
<tr>
<td>PABPC1</td>
<td>5'-GTCTTCTCCGGCTCAAAGGTG-3'</td>
<td>5'-GCTAGACCTGGCATTTGCT-3'</td>
</tr>
<tr>
<td>N-Myc</td>
<td>5'-GTCGTGAGTAGTGCTGGACAC-3'</td>
<td>5'-CTCGTCACTCCTCATGCT-3'</td>
</tr>
<tr>
<td>L-Myc</td>
<td>5'-CATGAAGCACTTCCCATATCTC-3'</td>
<td>5'-GTTCCTTCCTTGGTCACGT-3'</td>
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<tr>
<td>5'-ETS(^b)</td>
<td>5'-CTCTTCCCGGTCTTTTCC-3'</td>
<td>5'-TGATACGGCGACACAGGAA-3'</td>
</tr>
<tr>
<td>5S rRNA</td>
<td>5'-TGGTTTGTCGGAGCTGAGG-3'</td>
<td>5'-AAGGGAGGCGCTAAGGG-3'</td>
</tr>
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</table>

\(^a\) RP: ribosomal protein

\(^b\) 5'-ETS of the 45S rRNA precursor
Additional data: Image analysis

One major experimental approach of our study was the automated analysis of areas for quantification of alveolar area. It is worth mentioning the analysis process in more detail since much effort went into producing the accurate results published in the Research Article (Stoelzle et al., 2009). Automated staining of mammary gland sections was performed using Ventana Discovery System (program ‘mCC1’) with a CK18 antibody (Progen) to stain specifically the epithelium (alveoli and ducts) while nuclei (lymph node, stromal cells and epithelium) were counterstained in blue. Automated overnight scanning of the sections was done with a Mirax Scan slide scanner (Zeiss) that produced high resolution images (around 1 GB per slide) that could be viewed and zoomed in with the Mirax Viewer (Fig. S1).

![Figure S1: Mirax slide scanner (left) and Mirax Viewer (right). Easy and automated scanning produced perfectly stitched images of whole sections, which could be examined with the Mirax Viewer. The viewer allows easy zoom-in up to high magnification (40x).](image)

Images were exported from the Mirax Viewer into AxioVision in a lower resolution to allow image analysis, which was carried out using Image Pro, Image Access and Definiens software. The goal of the image analysis was to detect organ area, lymph node area and alveolar area (epithelium and lumen), in order to calculate the ratio of alveolar area over total organ area. A feasibility study performed on one
test slide using Definiens promised a high accuracy (Fig. S2) as a detailed detection of alveolar epithelium and lumen as well as the other organ components was achieved.

![Figure S2: Feasibility study with Definiens. A very detailed detection of different categories of areas was obtained on a test slide. Left: original scanned image. Right: segmentation performed by Definiens.](image)

However, when the large scale analysis was launched, we were confronted with different problems in alveolar detection and so that we used Image Pro and Image Access as two other approaches to validate the results obtained by Definiens. Two major problems that had to be solved with Definiens were the detection of blood vessels or other background features as ‘false positive’ alveoli and the detection of large alveolar areas as ‘false negative’ background (see Fig. S3). This led to big errors in the calculation of alveolar area, which became clear when comparing the values to the results obtained from manual quantification. Manual analysis was carried out with the ‘Measurement’ option in Image Access and manual ‘drawing’ around alveoli on a pen screen. This produced by far the most accurate results; however, quantification took between 1 and 6 hours per image, depending on the developmental stage of the animal.
Figure S3: Problems with Definiens. Automated detection of alveolar and stromal area caused big problems when glands were densely filled, like at lactation day 10.5. Due to wrong classification, alveolar area for this section resulted in 60% when calculated by Definiens (left panel), while manual measurement (right panel) resulted in 80%.

A very accurate analysis could also be performed with Image Pro, depending on the quality of the original image and staining. The analysis was based on a simple ‘color cube based’ segmentation. By picking a 3x3 or 5x5 pixels color field, alveolar epithelium was detected, and closed objects were generated by using the ‘spatial filtering’ option. This generated filled objects that could be automatically measured (counting and area measurement) and provided us with a relatively easy, fast and accurate alternative for area measurement. However, quality of the results was strongly depending on the quality of the staining and the developmental stage of the mammary gland with the biggest errors also occurring at lactation day 10.5. Only long and intense discussions with Definiens could solve all problems according to our satisfaction. This required many rounds of trouble-shooting and could only be accomplished with the tremendous support of Patrick Schwarb. The final analysis carried out by Definiens was highly accurate, could be applied to our large set of sections, and the results were completely in the range of manually obtained results. A summary of all 3 different methods and a comparison between Definiens and manual analysis is shown in Figure S4.
Figure S4: Quantification of alveolar area via different methods. (A) ImagePro, (B) manual measurement in ImageAccess and (C) automated measurement with Definiens. (D) For quality control, various sections of wild type (Wt) and mutant (Mu) glands from different time points of pregnancy and lactation were measured manually and values compared to the Definiens results. The chart shows the final results of the analysis.
In our study, we describe for the first time effects of c-Myc loss on normal mammary gland development using a conditional knockout approach. Our model is based on WAPiCre transgenic mice, in which recombination and loss of c-Myc specifically occurs in luminal alveolar cells starting in mid-pregnancy. Three major phenotypes were observed in c-Myc-deficient glands: first, during early pregnancy mutant cells displayed a slower proliferative response, leading to a delayed, but not blocked differentiation. Second, polysome fractionation revealed a decreased translation efficiency in mutant glands, which was accompanied by lower levels of ribosomal proteins and rRNA. This resulted in reduced milk production and slower body weight-gain of pups nursed by mutant mothers. Third, in transplantation experiments, epithelium from mutant glands showed less ability to repopulate a cleared fat pad, suggesting a role for c-Myc in progenitor cells. As this is the first study describing a CKO of c-Myc in the mammary gland, it is interesting to compare our results with other published reports and to highlight similarities as well as differences.

C-Myc had already been implicated in normal mammary gland development, from studies with transgenic mice (see Introduction Chapter V: Hynes and Stoelzle, 2009). During embryogenesis, Neuregulin3 (Nrg3) is an important signal for placode formation and ectopic expression of Nrg3 in K14-Nrg3 mice led to epidermal hyperplasia and formation of additional placodes (Howard, 2008). Interestingly, high levels of c-Myc and decreased levels of adhesion proteins were observed in the skin of K14-Nrg3 animals, suggesting that Nrg3 might regulate lineage commitment via c-Myc, by changing adhesion properties of the cells. In another study, transient overexpression of c-Myc from day 12.5 to day 15.5 of pregnancy induced precocious proliferation and differentiation (Blakely et al., 2005). The authors showed that this effect was due to the ability of c-Myc to downregulate Cav1, a negative regulator of the Prl-Jak2-Stat5 signaling. Finally, the third set of data implicating c-Myc in the
mammary gland suggested a role at involution; it was shown that overexpression of c-Myc prior to weaning resulted in accelerated involution and increased levels of pro-apoptotic proteins (Sutherland et al., 2006). The results suggested that c-Myc acts as a downstream mediator of Stat3 in promoting apoptosis in the mammary gland. A model of all possible c-Myc functions in normal mammary gland development is shown in Figure S5.

To which extent are these three main phenotypes discussed above (apoptosis, proliferation, adhesion properties, shown in green in Figure S5) recapitulated in the CKO used in our studies? Despite the reports that suggest a role for c-Myc in involution and apoptosis, we did not detect major changes during involution in c-Myc-deficient mice. However, it is possible that subtle effects might have been overlooked. It is difficult to study the complicated process of involution since the process can occur in a very non-uniform way. This is the case for inbred mouse strains and even more problematic in mice from a mixed background. In addition, it may well be that the mutant mice developed alternative pathways to compensate for c-Myc loss during involution.
Regarding the effects on proliferation and differentiation observed by Blakely et al., we did not detect changes in BrdU incorporation during the proliferative burst at early lactation. This is consistent with many other studies that found c-Myc to be dispensable for proliferation in vivo (Baena et al., 2005; Bettess et al., 2005; Dubois et al., 2008; Oskarsson et al., 2006; Wilson et al., 2004) and suggests that proliferation during lactation proceeds via different pathways. The stage where we did find slower or delayed proliferation in mutant mice was during early second pregnancy. As discussed in the Research Article, it is possible that the phenotype observed in second pregnancy results from the decreased translation rate during the preceding lactation in which c-Myc-deficient cells could not prepare properly for efficient proliferation. Another possibility is that c-Myc is required during this phase of proliferation downstream of progesterone signaling (as c-Myc levels are normally high during early pregnancy (Master et al., 2002)). We also examined the protein levels of Caveolin1 as it was shown to be a repressed c-Myc target and it was changed in the c-Myc overexpressing mammary glands (Blakely et al., 2005). Interestingly, while no consistent results were obtained during a first pregnancy (Fig. S6A), a higher percentage of mutant mice showed elevated protein levels of Cav1 during the second pregnancy (Fig. S6B) suggesting that this contributes to the strong phenotype observed. In agreement with Blakely et al. this would suggest that c-Myc indeed regulates differentiation by at least modulating Prl-Stat5 signaling via Cav1 expression.
Figure S6: Western analysis of Caveolin1, a direct repressed c-Myc target. (A) Mammary gland lysates from wild type (W) and mutant (M) mice obtained during first lactation were examined for Caveolin1 levels. Compared to the loading control α-tubulin, some mutant samples show higher levels of Caveolin1, marked with asterisks. (B) Caveolin1 levels in mammary glands of mice from second pregnancy and lactation. Although the loading is not equal, the difference in Caveolin1 is more striking than in first pregnancy. Caveolin1 seems to be higher in mutant mice from P(II)16.5 and L(II)3.5. Antibody: Caveolin1 (BD Bioscience, #610059).

Finally, to investigate the third main phenotype observed in other models, which is c-Myc’s effect on adhesion molecules, we performed Western analysis for β1-integrin, as this was shown to be affected in other c-Myc models (Howard, 2008; Wilson et al., 2004). There was again high variability between different animals, but some c-Myc mutant mice displayed higher protein levels of β1-integrin (Fig. S7). While this result needs to be confirmed in a larger number of animals, it is interesting to speculate that by changing the adhesive properties of epithelial cells, including the progenitor cells (Pi-MECs), c-Myc might influence migration of the cells and interaction with the surrounding niches, as shown for the hematopoietic stem cells (Wilson et al., 2004). As β1-integrin was previously shown to be important in mammary epithelial and progenitor cells (Li et al., 2005), deregulation of β1-integrin
levels could as well contribute to the phenotype seen in c-Myc-deficient glands, especially regarding the progenitor cells. In summary, some aspects of the phenotype observed in our model are partly found also in other c-Myc models, while others, such as apoptosis, seemed not to be affected by c-Myc loss.

Figure S7: Western analysis of β1-integrin, a repressed c-Myc target. (A, B) Mammary gland lysates taken from wild type (W), mutant (M) and heterozygous (H) mice at different time points of lactation were analyzed for β1-integrin levels. Compared to the loading control α-tubulin, some mutant mice show elevated protein levels of β1-integrin. Antibody: see (Graus-Porta et al., 2001).

It is worth mentioning two important general points that could be considered in further studies on c-Myc in the mammary gland. First, our model used WAPiCre, which is hormonally induced during pregnancy and its expression therefore not subject to ectopic induction. Some open questions that remain would eventually be better addressed using an inducible system like MMTV-rtTA/TetO-Cre, in which Cre expression and recombination are induced by doxycycline administration at any time point. This would allow a specifically regulated c-Myc loss, and would enable us to look separately at its effect on pregnancy, lactation and involution. A second
drawback is that the mice were in a mixed background, making it hard sometimes to compare even wild type littermates to each other. Using mice in a pure background like FVBN could reduce the variation seen between animals and enhance the phenotype even further. Nevertheless, novel and exciting functions for c-Myc were discovered with our model.

We consider that a very interesting aspect discovered in our study is c-Myc’s effect on translation, and the specificity within it. It is well known that c-Myc affects ribosome biogenesis and translation on multiple levels (see e.g. Oskarsson and Trumpp, 2005), but from the various KO studies performed in different organs, changes in translation were only detected in the intestines (Muncan et al., 2006). This demonstrates again that c-Myc affects different cell types/organs in different, specific ways: during lactation when the mammary gland is devoted to biosynthetic activity and secretion, efficient translation is probably the most essential process. By performing qPCR we observed that various c-Myc targets that are involved in ribosome biogenesis and translation (see Table 1 in research article) were expressed to lower levels in mutant compared to WT glands. This led us to the hypothesis that by lowering many of the components needed for translation, loss of c-Myc decreases the general translation efficiency in mutant glands, which is confirmed by our results from polysome fractionation. It would be interesting to further confirm the effect on ribosomal subunits and investigate ribosomal numbers, for example, via electron microscopy. This was not possible in a quantitative way on the pictures we obtained for our vesicle-studies. Higher resolution and eventual staining of ribosomes would be necessary to count them in WT and mutant cells.

One novel aspect was that c-Myc affects translation efficiency of specific targets, such as milk proteins or enzymes involved in milk synthesis, while translation of house-keeping genes stays relatively constant. The possible mechanisms behind this observation are worth being discussed in more detail. We think that three different mechanisms are plausible. First, as discussed in detail in the Research Article, changing the availability of ribosomes might affect translation of most abundant,
highly upregulated mRNAs whose products are involved in milk synthesis, while other mRNAs, such as house-keeping genes, which are not upregulated during lactation, remain translated in constant manner (Stoelzle et al., 2009).

Second, a new mechanism of how c-Myc regulates gene expression was recently found by Cole and Cowling (Cole and Cowling, 2009; Cowling and Cole, 2007). They show that c-Myc induces methylation of the 5’ guanosine cap of mRNA, generating m7G-mRNA, which is necessary for efficient translation. Interestingly, c-Myc induced cap methylation on 9 out of 10 analyzed targets, and this effect was in each case higher than the transcriptional increase in total mRNA (Cole and Cowling, 2009). Furthermore, they showed that the increased cap methylation led to a shift of those mRNAs to bigger polysomes, meaning that by methylating its target gene mRNAs, c-Myc enhances their translation potential. Levels of GAPDH mRNA and 7mG-mRNA were unresponsive to c-Myc and consequently mRNA distribution of GAPDH in the polysome profile was unchanged (Cole and Cowling, 2009). This means that traditional c-Myc targets (including also Scd, Fads2 and Elovl1) might be subject to c-Myc mediated cap methylation, and thereby shift to bigger polysomes. We hypothesize that this mechanism contributes to the polysomal shift we detected for some c-Myc targets, since their total mRNA levels between WT and mutant mice were unchanged. It would be essential to investigate the levels of 7mG-mRNA of those targets in c-Myc mutant and WT animals, to confirm whether c-Myc affects cap methylation in the mammary gland. In addition, the authors observed in their initial study that genes which are not described c-Myc targets were also affected by methylation changes, and higher protein levels were found while mRNA levels were constant (Cowling and Cole, 2007). Although this has yet to be shown, one can imagine that c-Myc regulates methylation of numerous ‘new’ targets (maybe including milk proteins?) that are not transcriptional targets.

Finally, there is a third possibility to achieve selective translation, which is via polyadenylation, a process highly investigated for milk proteins (for review see Rhoads and Grudzien-Nogalska, 2007). The poly(A) tail at the 3’end of mRNA is
another modification that strongly enhances translation initiation and mRNA stabilization. Milk proteins (most investigations concentrated on β-casein) were found to undergo various changes in poly(A) length, being predominantly polyadenylated and translated during lactation and subsequently becoming deadenylated. More recently, it was shown in CID9 cells that these changes were induced by lactogenic hormones (Choi et al., 2004). In this study, β-casein mRNA shifted to larger polysomes in response to insulin plus prolactin, while GAPDH mRNA was not affected. Furthermore, while total mRNA of β-casein did not increase, the poly(A) tail was found to be elongated and there was no effect on GAPDH poly(A) tail, suggesting that polyadenylation in response to hormones is indeed an important physiological stimulus of milk protein translation. How could loss of c-Myc interfere with this? To answer this question, it would be most important to analyze the poly(A) tails in the c-Myc mutant mice, to see if c-Myc is essential for the polyadenylation process itself, as a mediator of the yet unidentified pathways from insulin and prolactin receptor (model discussed in Rhoads and Grudzien-Nogalska, 2007). Another possible option is that c-Myc activates specific proteins that use the poly(A) tail to enhance translation efficiency and loss of c-Myc would result in decreased expression of those proteins. This would probably as well affect specifically mRNAs with long poly(A) tails, while mRNAs with short (or constant) poly(A) tails would, in theory, not be affected. An interesting candidate for this is the cytoplasmic poly(A) binding protein PABPC1, which is a direct c-Myc target, and was found in our model to be decreased in mutant mice. One of many functions of PABPC1 is binding poly(A) tails to ensure mRNA stability and formation of the translation complex; furthermore, more than one PABPC1 can bind on long poly(A) tails (Mangus et al., 2003). Thus, loss of c-Myc could result in insufficient PABP-occupation of poly(A) tails due to decreased levels of PABPC1, and it is possible that mRNAs which get highly polyadenylated during pregnancy and lactation are more sensitive to this change than mRNAs whose poly(A) tails do not change.
Another relevant point for discussion is the comparison of our results to other published studies that showed a similar phenotype in the mammary gland, and which models were used to generate it. In this respect we will concentrate on two reports, describing mice with full body KO of Akt1 (Boxer et al., 2006) and USF2 (Upstream stimulatory factor, Hadsell et al., 2003). The protein kinase Akt is interesting as it impacts on c-Myc protein levels via different mechanisms (Wierstra and Alves, 2008). Furthermore, Akt has been implicated in lipid metabolism in the mammary gland: expression of activated Akt induced a precocious and increased lipid synthesis in pregnant mammary glands (Schwertfeger et al., 2003). In contrast, KO of Akt1 resulted in a lactation defect with mutant mothers having reduced total milk volume, but comparable levels of milk proteins and milk lipid, leading to a reduced pup weight-gain (Boxer et al., 2006). Similar to what we observed in c-Myc-deficient glands, the Akt1 mutant glands contained the same number of alveoli as WT glands, but alveoli were less distended. The authors went on to show that there was no effect on proliferation, apoptosis and differentiation status in Akt1 KO animals. Instead they found that glucose uptake was less efficient in the mutant glands, due to a decreased expression of the glucose transporter Glut1. In addition, fewer vesicles were found to bud from the endoplasmic reticulum, less lipid droplets were detected in the cytoplasm, and several enzymes involved in lipid metabolism were mis-expressed in the Akt1-deficient glands. While an endocrine effect resulting from the full body KO cannot be excluded, it is still very interesting to discuss the similarities to the c-Myc CKO. Besides the lactation defect with reduced volume of otherwise identically composed milk, we observed a very similar pattern in the electron microscopy regarding the vesicles. Enzymes of the lipid metabolism were also affected in c-Myc mutant glands, although we did not detect expression changes but rather changes in translation efficiency. One striking point is the expression of Glut1, which is normally upregulated from virgin to lactating glands, and was shown to be regulated by Akt1, but is also a direct target of c-Myc (Slc2a1 on http://www.myc-cancer-gene.org/index.asp). This led us to test Glut1 expression on lactation day 10.5 in WT and mutant glands via immunofluorescence (Fig. S8).
Figure S8: Immunofluorescence for Glut1 on mammary glands. Frozen sections (10 µm) of mammary glands obtained on lactation day 10.5 were stained for the Glut1 glucose transporter. Every image belongs to a different animal, all four animals were littermates. There is a clear tendency of lower Glut1 expression in the mutant glands. Method: Slides were fixed in ice cold acetone for 5 min. After washing with PBS and permeabilization in 0.1% Triton/PBS (15 min) slides were blocked for 2h at RT with 10% goat serum/PBS. Sections were incubated with primary antibody Glut1 (Alpha Diagnostics, GT11A) overnight at 4°C. Secondary antibody: anti-rabbit FITC (Molecular Probes). Nuclear counterstain: Hoechst. Images were obtained on an Axio Imager Z1 microscope using an AxioCamMR3 camera. All images were acquired using the identical exposure time. Scale bar, 100 µm.

Indeed, in the analyzed mice there was a tendency for lower Glut1 expression in c-Myc mutant glands; however, the effect was not as strong as seen in the Akt1-deficient glands and the required staining of frozen sections hindered our ability to confirm the results in more animals. While c-Myc has not been addressed in the study of Boxer et al., it is nevertheless interesting to speculate that Akt1 regulates Glut1, glucose uptake and lipid synthesis in part via c-Myc.

Another phenotype sharing some similarities to what is observed in c-Myc-deficient glands was found in USF2 KO mice (Hadsell et al., 2003). This is worth
mentioning as USF2 is another B-HLH-LZ transcription factor related to c-Myc and can bind the same E-box element sequences; however, whether USF cooperates with or represses c-Myc mediated transcription is cell type and context dependent (Corre and Galibert, 2005). In USF2 KO animals, milk volume was decreased and milk contained lower levels of lactose, which is responsible for water influx into the milk vesicles (Hadsell et al., 2003). Furthermore, the authors observed a decrease in luminal alveolar area in the mutant glands and lower protein levels of the translation initiation factors eIF4E and eIF4G, which are known c-Myc targets. However, as these were not changed on the mRNA level in USF2-deficient mice, a direct (transcriptional) involvement of USF2 or c-Myc in this phenotype was questionable. We checked the mRNA levels of eIF4E or eIF4G via semi-quantitative RT-PCR in our c-Myc mutant glands, but we did not detect any consistent changes (data not shown). Since the USF2 KO was also a full body KO, like the Akt1 KO described above, a systemic function rather than a cell autonomous mechanism might have a role in the phenotype, for example via affecting blood oxytocin levels (Hadsell et al., 2003). Nevertheless, this study provided us with some good ideas and methods for studying milk and mammary glands in mice displaying a lactation defect.

One remaining aspect, which was not addressed in our study, is the connection between c-Myc and breast cancer. Breast cancer is still one of the leading causes of death in women in the developed countries and elevated protein levels of c-Myc are found in a large fraction (> 50%) of breast tumors (for more details, see Introduction Chapter V: Hynes and Stoelzle, 2009). Furthermore, c-Myc might play a prominent role in the basal breast cancer subtype, since a recent study found high transcriptional activity of Myc within this subtype (Alles et al., 2009) leading to the hypothesis that Myc maintains proliferation in those tumors independently of ER signaling (Musgrove et al., 2008). Even though targeting TFs is not trivial, there are several strategies for targeting oncogenic c-Myc (Ponzielli et al., 2005). As the effects of c-Myc loss on mammary gland physiology are relatively tolerable, making it theoretically a suitable target for inhibition, it would be interesting to investigate the
impact of c-Myc on tumor growth. This question has already been addressed in other studies, with diverse results. It was shown that c-Myc-deficient skin was resistant to Ras-mediated tumorigenesis (Oskarsson et al., 2006). Furthermore, in a Ras-induced lung carcinoma model, pharmacological inhibition of c-Myc triggered rapid regression on established tumors without irreversible side-effects (Soucek et al., 2008). In the mammary gland, there exists only one study that describes an effect of c-Myc loss on tumors, namely Notch-induced tumorigenesis. Using MMTV-Notch; myc$^{b/b}$; WAPCre mice it was shown that c-Myc was indispensable for the development of Notch-driven mammary tumor (Klinakis et al., 2006).

As discussed in Chapter V of the introduction, c-Myc is located downstream of multiple signaling pathways, such as Wnt, ErbB2 and Notch signaling (Fig. S9). In MMTV-Wnt1 and MMTV-Neu mice, which develop spontaneous mammary tumors, it will be interesting to investigate the role of c-Myc during tumor growth. As inhibition or loss of c-Myc shows variable effects in different tumor models, one could imagine that loss of c-Myc prior to tumor formation could increase the latency; alternatively loss of c-Myc in established tumor could lead to growth arrest and eventually to tumor regression. However, this remains to be examined in detail in the mammary gland. Interestingly it has recently been shown that c-Myc is an important effector of tumorigenesis driven by Apc loss in the intestines (Sansom et al., 2007), while it is totally dispensable after Apc loss in the liver where most Wnt targets were β-catenin dependent and c-Myc independent (Reed et al., 2008).
To conclude, our study suggests that c-Myc plays different important roles during mammary gland development which fall into 3 major functional areas: cell cycle, translation/metabolism and progenitor cells. Apart from a lactation defect, the effects of c-Myc loss are relatively mild and well tolerated as no apoptosis or dramatic tissue loss was observed. This is of special interest when considering c-Myc as a target in breast cancer therapy. Especially in the aggressive basal subtype of breast cancer, which cannot be treated with a targeted therapy like tamoxifen or trastuzumab, high pathway activation of c-Myc was found (Alles et al., 2009). Also, deregulation of c-Myc pathway was specifically detected in younger (<45 years) patients (Anders et al., 2008). In addition, apart from being a direct target of inhibition, c-Myc has been considered as a biomarker of basal breast tumors that might be able to predict response to certain chemotherapies (recently discussed in Bouchalova et al., 2009). Further studies and clinical trials will be necessary to clarify the importance of c-Myc as a therapeutic or predictive target in breast cancer.
References


# Abbreviations

## Abbreviations

### A

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Actb</td>
<td>β-actin</td>
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<tr>
<td>Ad</td>
<td>adeno</td>
</tr>
<tr>
<td>Aldo3</td>
<td>aldolase C</td>
</tr>
<tr>
<td>Apc</td>
<td>adenomatous polyposis coli</td>
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### B

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<tbody>
<tr>
<td>B</td>
<td>basic</td>
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<tr>
<td>BRCA1</td>
<td>breast cancer 1</td>
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### C

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<tr>
<td>Cav1</td>
<td>caveolin 1</td>
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<tr>
<td>Cbf1</td>
<td>C promoter-binding factor 1</td>
</tr>
<tr>
<td>Cdk</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>CK</td>
<td>cytokeratin</td>
</tr>
<tr>
<td>(C)KO</td>
<td>(conditional) knockout</td>
</tr>
<tr>
<td>Csn</td>
<td>casein</td>
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<tr>
<td>CTD</td>
<td>C-terminal domain</td>
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### D

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<td>DCIS</td>
<td>ductal carcinoma in situ</td>
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<tr>
<td>DMBA</td>
<td>7,12-dimethylbenz[a]anthracene</td>
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### E

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<td>E, ER</td>
<td>estrogen, estrogen receptor</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>Elovl</td>
<td>elongation of very long chain fatty acids</td>
</tr>
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<td>ERE</td>
<td>estrogen responsive element</td>
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<td>ETS</td>
<td>external transcribed spacer</td>
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### F

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<td>FACS</td>
<td>fluorescence activated cell sorting</td>
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<td>fatty acid desaturase</td>
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<td>FISH</td>
<td>fluorescence in situ hybridisation</td>
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<td>Fl</td>
<td>floxed</td>
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<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>GF</td>
<td>growth factor</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GH, GHR</td>
<td>growth hormone, growth hormone receptor</td>
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<tr>
<td><strong>H</strong></td>
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<tr>
<td>HLH</td>
<td>helix-loop-helix</td>
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<tr>
<td>HSC</td>
<td>hematopoietic stem cell</td>
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<tr>
<td><strong>I</strong></td>
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<tr>
<td>IC</td>
<td>intracellular domain</td>
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<tr>
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<td>interferon α</td>
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<tr>
<td>IGF-1</td>
<td>insulin-like growth factor 1</td>
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<td>IHC</td>
<td>immunohistochemistry</td>
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<td><strong>J</strong></td>
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<tr>
<td>Jak</td>
<td>janus kinase</td>
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<td>K</td>
<td>keratin</td>
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<td><strong>L</strong></td>
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<tr>
<td>Lαba</td>
<td>α-lactalbumin</td>
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<tr>
<td>LIF</td>
<td>leukemia inhibitory factor</td>
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<tr>
<td>LZ</td>
<td>leuzine zipper</td>
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<td><strong>M</strong></td>
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<td>M, Mu</td>
<td>mutant</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>Max</td>
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<td>Miz-1</td>
<td>Myc-interacting zinc finger protein 1</td>
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<td>MMP</td>
<td>matrix metalloprotease</td>
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<td>MMTV</td>
<td>mouse mammary tumor virus</td>
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<td><strong>N</strong></td>
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<td>NLS</td>
<td>nuclear localization signal</td>
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<td>open reading frame</td>
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<tr>
<td>P, PR</td>
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<tr>
<td>PABPC1</td>
<td>(cytoplasmic) poly(A)-binding protein 1</td>
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<td>PI3K</td>
<td>phosphatidyl-inositol 3 kinase</td>
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<td>parity-induced mammary epithelial cells</td>
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<td>PRE</td>
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<td>Prl, PrlR</td>
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<td>Pol</td>
<td>polymerase</td>
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<tr>
<td>R</td>
<td>Rank (L) receptor activator of NF-κB (ligand)</td>
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English: Fluent spoken and written
French: Basic knowledge

PUBLICATIONS

Stoelzle T, Schwarb P, Trumpp A, Hynes NE: c-Myc affects mRNA translation, cell proliferation and progenitor cell function in the mammary gland
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