MOLECULAR MECHANISMS OF ERBB2-DRIVEN THREE-DIMENSIONAL BREAST TUMOUR GROWTH.

By

Iman Aftab Khan

Submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

At

Dalhousie University
Halifax, Nova Scotia
August 2018

Dedicated to my parents

Late Dr. Aftab Ahmad Khan and Mrs. Fatima Bisquerra Khan

For their unconditional love, patience and struggle to support my decisions that helped me realize my dreams.

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ABSTRACT

The ability of breast cancer cells to survive anchorage-independently is thought to be a critical pre-requisite for breast carcinoma progression. Normal breast epithelial cells grow *in vivo* attached to the extracellular matrix (ECM). Detachment from the ECM causes their apoptosis, a phenomenon called as *'anoikis'*. Unlike normal breast epithelial cells, breast cancer cells remain viable after detaching from the ECM. This viability is critical for the ability of breast cancer cells to form three-dimensional tumours, invade other tissues and spread through the body. ErbB2/Her2 receptor tyrosine kinase is overproduced by 10-30% of breast cancers and is thought to play a critical role in their progression. ErbB2 blocks death of breast cancer cells detached from the ECM by partially understood mechanisms. In an effort to understand these mechanisms better we found that detachment of non-malignant breast epithelial cells causes upregulation of a transcription factor Irf6 which results in a further upregulation of a pro-apoptotic protein Perp. We further observed that Irf6 and Perp upregulation contributes to anoikis of these cells. We also noticed that ErbB2 blocks anoikis of breast cancer cells by downregulating Perp and Irf6 in a manner dependent on the activity of the mitogen-activated protein kinases (MAPK), which are well known to mediate ErbB2 signalling. Moreover, we observed that treatment with ErbB2-targeted drugs, such as an anti-ErbB2 antibody trastuzumab or an ErbB2/EGFR small molecule inhibitor lapatinib upregulates Perp and Irf6 in ErbB2-positive human breast cancer cells. In addition, ectopic expression of either Perp or Irf6 triggered anoikis of ErbB2-overproducing breast epithelial cells and blocked their anchorage-independent growth. We further found that detached ErbB2-overproducing breast cancer cells require the activity of a protein kinase Mek a major activator of the MAPKs, to block lysosomal degradation of ErbB2. We demonstrated that this effect of Mek on ErbB2 is required for anoikis resistance of breast cancer cells. Thus, we have identified novel molecular mechanisms by which ErbB2 promotes three-dimensional growth of breast cancer cells. These mechanisms are driven by ErbB2-induced activation of MAPK signalling, stabilization of ErbB2 protein in detached breast cancer cells and further ErbB2-dependent downregulation of Perp and Irf6.

LIST OF ABBREVIATIONS USED

ATP	Adenosine triphosphate
ALGGEN	Algorithmic and Genetics
α	alpha
ATCC	American Type Culture Collection
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
Apo2L	Apo2ligand
APAF1	Apoptotic protease activating factor 1
RGD	Arginine-Glycine-Aspartate
att	attached
BLNK	B-cell linker
Bcl-XL	B-cell lymphoma-extra large
Bcl-2	B-cell lymphoma 2
Bad	Bcl-2 associated agonist of cell death
Bax	Bcl-2 associated X protein
Bak	Bcl-2 homologous antagonist killer

BH	Bcl-2 homology
Bim	Bcl-2 interacting mediator of cell death
Bmf	Bcl-2 modifying factor
β	beta
Bid	BH-3 interacting-domain death agonist
BimEL	Bim extra-long
BPE	bovine pituitary extract
BSA	Bovine Serum Albumin
BCSC	Breast cancer stem cells
BES	N, N-bis (2 hydroxyethyl)-2-aminoethanesulfonic acid
CBF1	C-repeat/DRE binding factor 1
Cbl	Casitas B-lineage Lymphoma
CSL	CBF1, Suppressor of Hairless, Lag-1
Cdk4	Cyclin-dependent kinase 4
CHX	cyclohexamide
CMV	Cytomegalovirus

DR	Death Receptor
Δ	Delta
DNA	deoxyribonucleic acid
det	detached
DAG	Diacyl glycerol
DMEM	Dulbecco's Modified Eagle Medium
DMEM/F12	Dulbecco's Modified Eagle Medium F12
EGR3	early growth response gene
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
E-cadherin	Epithelial-cadherin
EMT	epithelial-mesenchymal transition
ErbB	erythroblast leukemia viral oncogene
ER	estrogen receptor
ECM	Extracellular matrix
ECD	extracellular-ligand binding domain
ERK	extracellular-signal regulated kinase

ERO-1	Endoplasmic reticulum oxidoreductin-1
FasL	Fas ligand
FADD	Fas-associated death domain
FAO	fatty acid oxidation
FACS	Fluorescence activated cell sorting
FAK	pp125 focal adhesion kinase
FISH	Fluorescence In Situ Hybridization
Glucose 6-P	Glucose 6-phosphate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
Grb2	Growth factor receptor-bound protein 2
GDP	gunosine diphosphate
GTP	gunosine triphosphate
GSH	glutathione reduced
GSSG	oxidized glutathione

H-Ras	Harvey-Ras
HSP90	Heat shock protein 90
HA	hemagglutinin
h	hour
Hek293T	Human Embryonic Kidney 293 SV40 large T antigen
hEGF	human epidermal growth factor
Her	Human epidermal growth factor receptor
HMEC	human mammary epithelial cells
HIF-1	Hypoxia inducible factor-1
IAP-1	inhibitor of apoptosis-1
IGF1R	type-1 insulin-like growth factor receptor
IP3	Inositol triphosphate
IRS-1	insulin receptor substrate-1
Irf6	Interferon regulatory factor 6
IRAK	Interleukin-1 receptor-associated

	kinase
JAK	Janus kinases
JNK	Jun-N terminal kinases
KRT23	keratin 23
K-Ras	Kirsten-Ras
LMP	lysosomal membrane permeabilization
mTOR	mammalian target of rapamycin
MEGM	Mammary epithelial cell growth medium
MEBM	Mammary Epithelium Basal Medium
MMP	matrix metalloproteinases
mRNA	messenger ribonucleic acid
MCF10A	Michigan Cancer Foundation
miRNA (miR)	microRNA
MEM	Minimal essential medium
miRSC	miRNA-induced silencing complex
MOMP	mitochondrial outer membrane

	permeabilization
MAPK	mitogen-activated protein kinase
Mek	mitogen-activated protein kinase kinase
MLKL	mixed-lineage kinase domain-like protein
MMTV	mouse mammary tumour virus
NDRG2	N-Myc downstream-regulated gene 2
N-Ras	Neuroblastoa-Ras
NADPH	nicotinamide adenine dinucleotide phosphate
NICD	Notch intracellular domain
NF-kb	nuclear factor kappa-b
OMM	Outer mitochondrial membrane
Perp	p53 apoptosis effector related to PMP-22
PERK	protein kinase R (PKR)-like endoplasmic reticulum kinase
p90RSK	p90 Ribosomal s6 kinase

P-values	Pearson-values
PPP	Pentose phosphate pathway
PMSF	phenylmethylsulfonyl fluoride
PTEN	phosphatase and tensin homolog
PBS	phosphate buffer saline
PI3K	phosphatidyl inositol 3 kinase
PAM	Phosphatidylinositol 3-kinase/Akt/mTOR
PI3KCA	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform
pRsk	phospho-RSK
PDI	Protein disulfide-isomerase
PDK	phosphoinositide-dependent kinase
PDK4	Pyruvate dehydrogenase kinase 4
PDH	Pyruvate dehydrogenase
PLC-γ	phospholipase C-gamma
PTB	phosphotyrosine binding domain

PCR	Polymerase chain reaction
PVDF	Polyvinylidene difluoride membrane
FAK	pp125 focal adhesion kinase
PR	progestrone receptor
PRSS8	protease serine S1 family member 8
p53	protein 53
PKB	Protein kinase B
PKC	Protein Kinase C
qPCR	quantitative polymerase chain reaction
Raf	Rapidly Accelerated Fibrosarcoma
RASSF5	Ras association domain family member 5
Ras	Rat Sarcoma
ROS	reactive oxygen species
RIPK	receptor interacting protein kinases
RTK	Receptor tyrosine kinase
rRNA	ribosomal RNA
RPMI	Roswell Park Memorial Institute

Src	Sarcoma
STAT	signal transducer and activator of transcription
SOD1	Superoxide dismutase 1
SOD2	Superoxide dismutase 2
siRNA	small interfering RNA
NaCl	Sodium chloride
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sos	son of sevenless
SGPP2	sphingosine-1-phosphate phosphatase 2
SFK	Src family of protein kinases
SH-2	Src homology 2
SHC	Src homology 2 domain-containing
SH-3	Src homology 3
SD	Standard deviation
TNC	Tenascin-C

TDLU	Terminal ductal lobular units
TEMED	Tetramethylethylenediamine
TIMP3	Tissue inhibitor of metalloproteinase 3
TRAIL	TNF-related apoptosis-inducing ligand
TA	Transactivation
TBST	Tris buffer saline with Tween-20
TNF-α	Tumour Necrosis Factor-α
TNFR	Tumour Necrosis Factor-α receptor
TP63	Tumour protein 63
TACSTD2	tumour-associated calcium signal transducer 2
TrkB	Tyrosine receptor kinase B
UPR	Unfolded protein respone
YAP1	Yes associated protein
Zfp36	Zinc Finger Protein 36

ACKNOWLEDGEMENTS

I would like to acknowledge all the people for their contributions towards my graduate studies and for the completion of this thesis.

The first and the foremost person to whom I would like to express my sincere gratitude is my supervisor Dr. Kirill V. Rosen. I want to thank him for not only giving me an opportunity to work with him but also for his patience, motivation and enthusiasm. His intellectual vigor had helped me refine my research skills by virtue of which I was able to accomplish all my research goals in time. I could have never finished this thesis without his timely support and experience. I have gained immense knowledge and scientific experience in Dr. Rosen's lab which will further bolster my career in the right direction and thus for that, I truly am grateful to him.

I would also like to thank my committee members: Dr. Patrick Lee, Dr. Catherine Too, Dr. David Waisman and Dr. Graham Dellaire for the discussions and indispensable suggestions that helped me improve the quality of my research and also the skills of critically analysing my data. Thank you so much for supporting me throughout my work. Furthermore, I would like to express my thankfulness to the graduate co-ordinators: Roisin McDevitt and Dr. Jan Rainey for supporting me and helping me through the toughest situations. I cannot thank you all enough for your time, patience and support which I deeply needed.

I also owe a great deal of gratitude to Dr. Byong Hoon Yoo (a research associate in Dr. Rosen's lab) for his tremendous experience in the field of cancer cell molecular biology and biochemistry. Without his support I would have never been able to finish my manuscripts on time. I am very much thankful to Dr. Yoo for guiding me through my degree to not only become a better researcher but to also to become a better person. He taught me to be resilient, persistent, focussed and honest to my own self and to the work. I feel highly indebted for his patience and support.

I would also like to thank our previous and current lab technicians Robert Zwicker and Robert Douglas for taking care of all the cell lines that I have used during my research. I appreciate their time and effort to maintain the cell lines as my research truly depended on the availability of these cells. I am also thankful to all the ARC members for morally supporting me and for giving me useful expert advices on problems related to various aspects of my research. I deeply am thankful to our administrative secretary Debbie Hayes. Debbie had been a motherly figure. She encouraged me to fight back whenever time was not in my favor. For her unconditional love and kindness, I shall always feel overwhelmed.

Most importantly, I would like to thank the funding bodies Canadian Breast Cancer Foundation (CBCF), Canadian Cancer Society (CCS) and Breast Cancer Society of Canada (BCSC) for financially supporting my research.

I want to express my heartfelt gratitude to all my friends in Halifax and back home in India for emotionally supporting me at every step of my journey. I want to especially thank Astad Canteenwala for being more than just a friend to me. He had been the strongest support in times of great uncertainties. He made me believe that I am special and that I have potential to reach greater heights in life. Thank you for unconditionally caring for me. For his kindness, I shall ever remain indebted. Furthermore, I would like to acknowledge the fact that Astad helped me format this thesis. I would also like to thank Justin George for being the truest friend that I always needed. Justin's selflessness inspired me in many ways to become a better person. His ability to solve problems and critically analyse situations helped me improve my decision-making skills. Astad and Justin instilled confidence in me to achieve bigger goals. I feel more than lucky to have them as strong pillars supporting me through various struggles of my life.

Last but never the least, I would like to thank my family for being there for me whenever I needed them the most. My parents made many sacrifices to have me achieve my goals. They never stopped believing in me. They picked me up each time whenever I had fallen due to hopelessness. They always trusted my decisions in building my own individuality. They provided me with the wings to fly high. My father Late Dr. Aftab Ahmad Khan strongly believed in my potential, respected my decisions and gave me the freedom to follow my passion. I miss him, and I wish he could see me crossing the finish line. I also want to thank my mother Mrs. Fatima Bisquerra Khan for being my role model. Her quality of perseverance inspired me to be who I am. She taught me to never give up. She taught me the significance of being an independent strong woman. She taught me to be the woman of great self-esteem but yet humble at the same time. I also want to thank my brothers Mohammad Aftab Khan and Sultan Aftab Khan for their emotional and moral support despite the long distances between us. I would also like to thank Dr. M. M. A. Faridi my uncle who believed in my ambitions and thus helped me take the path towards achieving this goal. Due to him I was able to gain first class molecular biology training at the National Institute of Immunology, New Delhi, India and hence gained future interests in learning more about cancer cell biology.

Thank you all for everything…. And in the end with my ever-growing trust in God Almighty I would like to mention the following quote:

"The best form of worship is the pursuit of knowledge."

- Prophet Muhammad (peace be upon him).

CHAPTER 1. INTRODUCTION

Resistance to detachment-induced cell death as a critical feature of ErbB2-driven breast cancer: an overview of project goals.

The goal of the project is to identify the mechanisms by which ErbB2 oncoprotein promotes three-dimensional breast tumour growth. Human breast epithelium is composed of two epithelial layers, an inner luminal layer (Gudjonsson et al., 2002; Weaver et al., 2002) and an outer myoepithelial layer (Gudjonsson et al., 2002). The myoepithelial cells produce the extracellular matrix (ECM) to which the luminal cells are attached (Gudjonsson, Adriance, Sternlicht, Petersen, & Bissell, 2005; Gudjonsson et al., 2002). The myoepithelial cell layer is in turn attached to a form of ECM known as the 'basement membrane' (Gudjonsson et al., 2005; Hassiotou & Geddes, 2013; Nelson & Bissell, 2005). ECM is a meshwork of various structural glycoproteins such as collagen, laminins and fibronectins to which the normal epithelial cells attach for survival (Bosman & Stamenkovic, 2003). Detachment of breast epithelial cells from the ECM kills them by apoptosis, a phenomenon known as "anoikis" (which means "homelessness" in Greek) (Frisch & Francis, 1994.). Anoikis plays an important role in mediating morphological changes within mammary gland during various stages of development. For instance, anoikis is thought to promote lumen formation within the mammary duct (Grossmann, 2002a; Muschler & Streuli, 2010).

Unlike normal epithelial cells, carcinoma cells are capable of surviving without adhesion to the ECM as three-dimensional disorganized multicellular tumour masses

(Debnath & Brugge, 2005). The cells within these tumours lack proper cell-ECM contacts but yet remain viable (Debnath & Brugge, 2005; S. M. Frisch & Screaton, 2001). It is known that these tumour cells produce ECM-degrading enzymes such as matrix metalloproteinases that are required for tumour invasion into the surrounding tissues (Ljubimov et al., 1992). Moreover, cancer cells are known to detach from their primary tumours, disseminate and form metastases at an ectopic site (Berezovskaya et al., 2005; Douma et al., 2004).

Numerous data suggest that anoikis resistance of cancer cells is a critical pre-requisite for tumour progression. For instance, the ability of cancer cells to grow without adhesion to the ECM and form colonies in soft agar is considered to be a "gold standard" for malignant transformation (Freedman & Shin, 1974). Moreover, we and other research groups have observed that activation of oncogenes such as ErbB2 (Haenssen et al., 2010; M. J. Reginato et al., 2003), EGFR (Liu et al., 2005; Rosen, Coll, Li, & Filmus, 2001) and Ras (Derouet et al., 2007; Liu et al., 2006; Liu et al., 2005; Kirill Rosen et al., 2000) can rescue various types of epithelial cells from anoikis. Finally, it was demonstrated that treatments aimed at inhibiting anoikis resistance of tumour cells block their ability to form tumours (Coll, Rosen, Ladeda, & Filmus, 2002; Duxbury, Ito, Zinner, Ashley, & Whang, 2004; Frankel, Rosen, Filmus, & Kerbel, 2001; H. Li, Ray, Yoo, Erdogan, & Rosen, 2009; Rosen et al., 1998; K. Rosen et al., 2000) and metastasis (Berezovskaya et al., 2005; Duxbury et al., 2004) in mice. Hence, the mechanisms of anoikis resistance of cancer cells is critical for cancer progression and represents a potential target for cancer therapies (Douma et al., 2004; S. M. Frisch & Screaton, 2001; Jacks & Weinberg, 2002).

ErbB2/Her2 oncoprotein is a receptor tyrosine kinase overproduced in 10 - 30% of breast cancers (Slamon, Godolphin, Jones, Holt, Wong, Keith, Levin, Stuart, Udove, & Ullrich, 1989). It is known to induce anoikis resistance of breast cancer cells through molecular mechanisms that are understood in part (Haenssen et al., 2010; M. J. Reginato et al., 2003). The goal of my project is to understand these mechanisms better.

1.1 The role of the cell-ECM interactions in the mammary gland development and functions.

ECM is an insoluble network of polysaccharides and proteins secreted by the overlaying epithelial cells that form a tissue (Bosman & Stamenkovic, 2003). ECM not only provides structural and mechanical support to these cells but also transmit extracellular signals to the cells to activate various biological processes such as tissue morphogenesis, differentiation and homeostasis (Ford & Rajagopalan, 2017; Walters & Gentleman, 2015; Zollinger & Smith, 2017). ECM proteins interact with the transmembrane cell adhesion receptors such as integrins to stimulate molecular mechanisms that regulate cell adhesion, migration, survival, proliferation and differentiation (Barker, 2011; Meredith, Fazeli, & Schwartz, 1993; Walters & Gentleman, 2015). It was observed that the breast epithelial cells cultured in contact with the ECM survived in the absence of growth factors in the serum (Pullan et al., 1996). This suggested that the components of the ECM provide cues for survival to the breast epithelial cells.

1.2 Morphology of the Human Mammary Gland.

The human mammary gland is composed of functional secretory units consisting of a highly organized branched network of mammary ducts ending with the clusters of alveoli, also known as terminal ductal lobular units (TDLU). The function of TDLUs is to generate and secrete milk. Each duct or lobule is composed of two epithelial layers. The inner layer of polarized cells is known as the luminal layer which is responsible for milk secretion. The apical region of the luminal cells faces the inner lumen of the duct whereas the basal region of the cells is in contact with an outer layer of breast epithelial cells known as the myoepithelial layer ((Figure 1) (Gudjonsson et al., 2002; Weaver et al., 2002). The myoepithelial layer is separated from the surrounding stroma by a form of the ECM known as the basement membrane (Gudjonsson et al., 2005; Hassiotou & Geddes, 2013; Nelson & Bissell, 2005). The myoepithelial cells attach to the basement membrane through multiprotein complexes known as the hemidesmosomes (M. C. Adriance, J. L. Inman, O. W. Petersen, & M. J. Bissell, 2005; Pitelka, Hamamoto, Duafala, & Nemanic, 1973).The contractile properties of myoepithelial cells allow the secreted milk to flow through the mammary ducts (Murrell, 1995). The myoepithelial cells have been reported to direct polarization of the luminal cells by secreting glycoproteins such as laminin-1 to which the latter cells attach through the cell surface receptors called 'integrins' (Gudjonsson et al., 2002). Disruption of such polarity has been shown to increase sensitivity of breast epithelial cells to apoptosis induced by activation of death receptors or treatment of cells with cytotoxic drugs (Weaver et al., 2002).

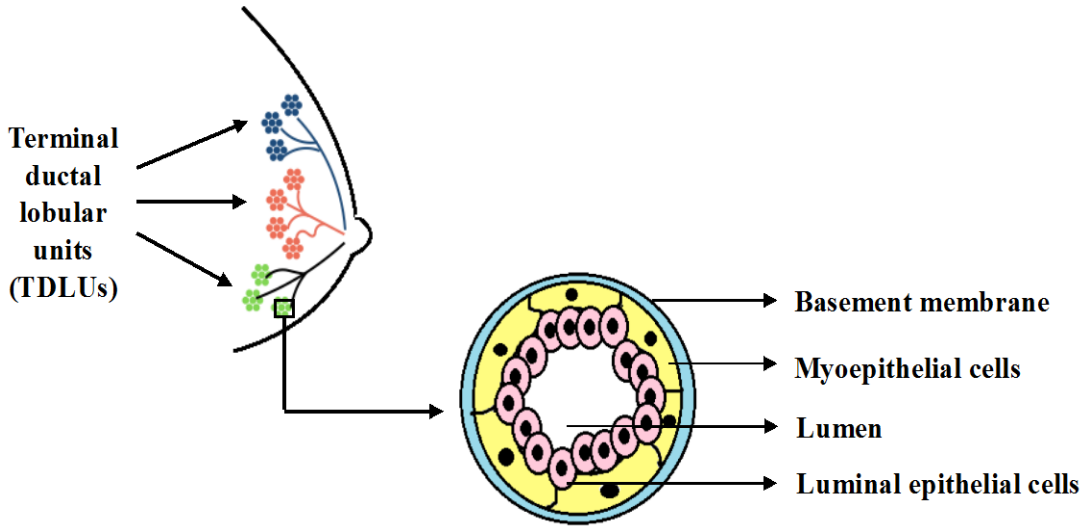

Figure 1. Cross-section of a bi-layered mammary duct. A mammary duct is composed of two epithelial layers: The luminal epithelial layer that surrounds an inner lumen and the myoepithelial layer that surrounds the luminal layer. The myoepithelial layer is surrounded by a form of the ECM known as the basement membrane (Melissa C. Adriance, Jamie L. Inman, Ole W. Petersen, & Mina J. Bissell, 2005).

Numerous lines of evidence have demonstrated the existence of self-renewing and undifferentiated breast stem cells across the mammary gland (Dontu, Al-Hajj, Abdallah, Clarke, & Wicha, 2003). Breast stem cells can act as progenitors of the luminal or the myoepithelial cells within a developing mammary gland (Dontu, Al-Hajj, et al., 2003). Unlike breast epithelial cells, the breast stem cells can survive without adhesion to the ECM and form mammospheres *in vitro* (Dontu, Al-Hajj, et al., 2003). Furthermore, it has been suggested that breast stem cells accumulate multiple transforming mutations that could eventually result in the development of breast carcinoma (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003).

1.3 The role of cell-ECM proteins in controlling breast epithelial cell survival.

1.3.1 The role of laminins in breast epithelial cell survival.

Laminins are a family of multifunctional high molecular weight glycoproteins that are present abundantly in the underlying basement membrane of the breast epithelium (Aumailley & Smyth, 1998). They are also secreted by other epithelial cell types such as that of muscle as well as by endothelial cells and are required to promote survival of these cells (Aumailley & Smyth, 1998; Vachon, Loechel, Xu, Wewer, & Engvall, 1996).

One tissue culture model often used to study the breast epithelium is based on the mammary acini, three-dimensional spherical structures formed by a single layer of epithelial cells surrounding an inner lumen cultured in a reconstituted basement membrane to recapitulate a mammary acinus (or alveolus) found *in vivo* (Barcellos-Hoff, Aggeler, Ram, & Bissell, 1989; L. O'Brien, Zegers, & Mostov, 2002). It was found that the breast epithelial cells were able to form mammary acinus due to their interactions with laminin-1 in the basement membrane (Gudjonsson et al., 2002). Such interaction with laminins is considered essential for survival of the luminal cells within the mammary acinar structures formed *in vitro* (Farrelly, Lee, Oliver, Dive, & Streuli, 1999; Weaver et al., 2002). It was shown that inhibition of attachment of the breast epithelial cells to laminins by the use of anti-laminin antibodies increased apoptosis of these cells (Farrelly et al., 1999; Pullan et al., 1996). Furthermore, treatment of mice with anti-laminin antibodies significantly suppressed formation of terminal end buds (functional equivalent of TDLUs in human mammary gland) during mammary gland development

(Klinowska et al., 1999). Hence, the data suggests that laminins play an essential role in promoting survival of breast epithelial cells.

1.3.2 The role of integrin-ECM interactions in breast epithelial cell survival.

Integrin-ECM interactions are required to regulate biological processes such as cell adhesion, survival or apoptosis required for proper functioning of the mammary gland (Ford & Rajagopalan, 2017; Walters & Gentleman, 2015). Integrins are heterodimeric transmembrane proteins that consist α and β subunits. 18α and 8β subunits assemble to form 24 possible conformations of integrins (R. O. Hynes, 2002). The extracellular domain of integrin binds to a specific amino acid sequences, such as Arg-Gly-Asp (RGD) peptide present in the ECM proteins such as collagens, laminins and fibronectins (Ruoslahti, 1996). Activated ligand-bound integrins recruit cytoskeletal adaptor proteins such as talin, paxillin and vinculin to the integrin cytoplasmic tails which recruits multiple focal adhesion kinases (FAK) (Calderwood, 2004; Nikolopoulos & Turner, 2002; Priddle et al., 1998; Subauste et al., 2004). The FAK molecules cross phosphorylate each other at the specific tyrosine residues which act as docking sites for non-receptor Src tyrosine kinases (Vuori, Hirai, Aizawa, & Ruoslahti, 1996). Activated Src kinases further phosphorylate FAK on additional tyrosine residues that act as docking sites for other adaptor proteins responsible for induction of various survival signaling pathways (Cary & Guan, 1999).

Several studies have demonstrated the significance of interactions between β1/β4 integrin subunits and the ECM proteins in promoting breast epithelial cell survival (Prince et al., 2002). It was observed that inhibition of cell-ECM attachment via treatment

of breast epithelial cells with the antibodies against β1 integrin induces apoptosis of these cells and suppresses mammary acinus formation *in vitro* (Farrelly et al., 1999; Howlett, Bailey, Damsky, Petersen, & Bissell, 1995; Pullan et al., 1996). Others observed that β4 integrin mediates activation of a transcription factor nuclear factor kappa-b (NF-kb) to promote apoptosis resistance of the breast epithelial cells surrounding the lumen and thus maintain the three-dimensional structure of the mammary acinus *in vitro* (Weaver et al., 2002). Recent studies also showed that increased expression of β4 integrin in nutrient-deprived breast epithelial cells (a situation commonly observed in detached breast epithelial cells (Schafer et al., 2009)) is required for internalization and lysosomal degradation of laminins to generate amino acids required for the survival of the indicated cells (Muranen et al., 2017).

In addition, it was demonstrated that expression of dominant-negative form of β1 integrin under the control of mouse mammary tumour virus (MMTV) in mice increased apoptosis of the cells and disrupted normal mammary gland development (Faraldo, Deugnier, Lukashev, Thiery, & Glukhova, 1998; Naylor et al., 2005). Moreover, treatment of mice with function blocking β1 integrin antibodies was observed to perturb cell-ECM adhesion and suppressed formation of terminal end buds in developing mouse mammary gland (Klinowska et al., 1999). Lack of ligand-bound β1 integrin during mammary gland involution was also suggested to promote breast epithelial cell apoptosis in mice (Prince et al., 2002). In conclusion, integrin-ECM protein interactions are considered essential to regulate breast epithelial cell survival during mammary gland development.

1.4 Physiological significance of 'anoikis' of breast epithelial cells in the mammary gland development and functions.

1.4.1 The role of anoikis in lumen formation within the mammary ducts.

Anoikis of breast epithelial cells was thought to contribute to the mammary duct lumen formation required for collection of milk (Grossmann, 2002b; Humphreys et al., 1996b). It was shown that the early stages of duct formation during mammary gland development in mice required clearance of centrally located inner cells via apoptosis of breast epithelial cells within the terminal end buds (Humphreys et al., 1996b). This event was found to occur in a p53-independent manner but yet was abrogated by expression of anti-apoptotic protein Bcl-2 (see section 1.5.1, page 10 for detailed description) (Humphreys et al., 1996b). Similar observations were made by Debnath et al., that the inner breast epithelial cells located away from the basement membrane died due to apoptosis to form a hollow lumen within the three-dimensional mammary acini structures *in vitro* (Debnath et al., 2002). Hence, it was suggested that detachment-induced apoptosis plays a significant role in formation of mammary duct lumen in the mammary gland (Figure 2).

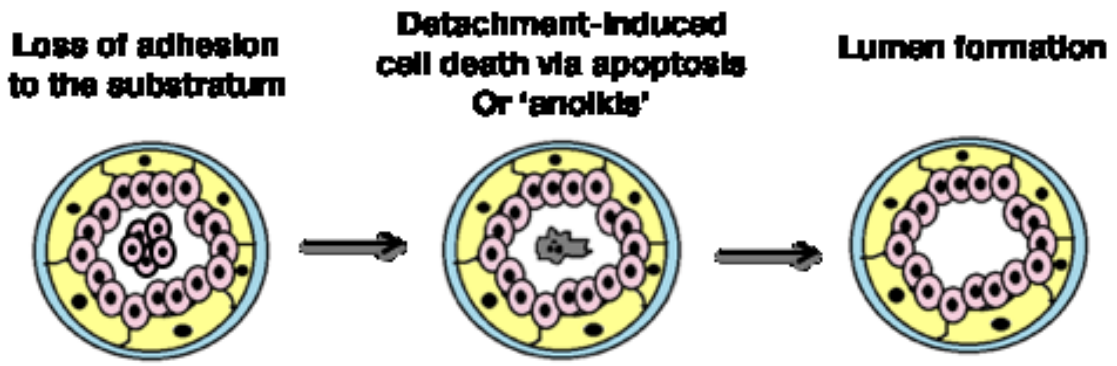

Figure 2. Schematic representation of detachment-induced breast epithelial cell death. Detached centrally located breast epithelial cells away from the basement membrane undergo anoikis to form a hollow lumen within the three-dimensional mammary acinus cultured *in vitro*. The dying cells at the centre of the lumen displayed hallmarks of apoptosis such as fragmented DNA and cleaved caspase-3 (Debnath et al., 2002; Grossmann, 2002a; Humphreys et al., 1996a).

1.4.2 The role of anoikis during involution process of the mammary gland.

During the post-lactation stage, the developed mammary gland undergoes a process of involution which involves clearing out of the milk secreting luminal cells from the alveoli of the mammary gland (Humphreys et al., 1996a). It was suggested that during advanced stages of involution, the mammary epithelial cells secrete ECM degrading enzymes such as matrix metalloproteinases (MMPs) that result in detachment of cells from the ECM followed by apoptotic cell death (Green & Lund, 2005; Lund et al., 1996; Strange, Li, Saurer, Burkhardt, & Friis, 1992a). Furthermore, expression of matrix metalloproteinase inhibitor TIMP3 (Tissue Inhibitor of Matrix metalloproteinase 3) was essentially found to regulate MMP-induced apoptosis of detached breast epithelial cells during involution. It was observed that the ablation of TIMP3 expression in mouse

mammary gland accelerated the process of involution via induction of early apoptosis of breast epithelial cells leading to early regression of the mammary gland (Fata et al., 2001). Therefore, it can be suggested that deregulation of signaling mechanisms controlling involution of the mammary gland can potentially contribute towards formation of breast carcinoma (Henson & Tarone, 1993; Lyons et al., 2011; Zaragoza, Garcia-Trevijano, Lluch, Ribas, & Vina, 2015).

Notably, anoikis has been proposed to represent a general mechanism of the formation of various cavities in the organism, e.g. embryonic cavitation or formation of the gut lumen was found to involve apoptotic cell death of embryonic or intestinal cells respectively that are located away from the basement membrane (Coucouvanis & Martin, 1995; Strange, Li, Saurer, Burkhardt, & Friis, 1992b).

Hence, anoikis is likely essential for the maintenance of tissue homeostasis as it prevents growth of detached epithelial cells. It represents an important mechanism of tumour suppression as it regulates survival of epithelial cells dependent on ECM adhesion.

1.5 Molecular mechanisms of breast epithelial cell death after detachment from the ECM.

1.5.1 Molecular mechanisms of anoikis.

Anoikis is a form of apoptosis (Frisch & Francis, 1994; Kerr, Wyllie, & Currie, 1972). In general, anoikis can be executed by two different molecular mechanisms:

intrinsic and extrinsic (S. M. Frisch & Screaton, 2001; A. P. Gilmore, 2005; Grossmann, 2002b).

The intrinsic pathway is activated by disruption of the mitochondrial function resulting into cytochrome c release in the cytoplasm which binds to the apoptotic protease activating factor or APAF1 to form a complex known as the 'apoptosome' (Riedl & Shi, 2004). The apoptosome recruits and activates pro-caspase 9 which further activates effector caspases-3 and -7. The effector caspases induce apoptosis by promoting proteolytic degradation of cellular substrates such as focal adhesion kinases, protein kinase B/Akt and receptor tyrosine kinases that are commonly known to promote cell survival (Desagher & Martinou, 2000; Elmore, 2007; Kurokawa & Kornbluth, 2009). The extrinsic pathway, on the other hand, is initiated by activation of transmembrane death receptors such as Fas, Tumour Necrosis Factor-α receptor (TNFR) and Death receptors (DR) 4 and 5 by binding to the extracellular death ligands such as Fas ligand (FasL), Tumour Necrosis Factor-α (TNF-α) and Apo2ligand(Apo2L)/TNF-related apoptosis-inducing ligand (TRAIL) respectively (Ashkenazi, 2002; Locksley, Killeen, & Lenardo, 2001). Once activated, death receptors activate initiator caspases-8 and -10 through an adaptor protein known as the Fas-associated death domain (FADD) (Ashkenazi, 2002). Activated initiator caspases activate effector caspases-3 and -7 which eventually trigger cell death (Grossmann, 2002b; R. C. Taylor, Cullen, & Martin, 2008; Wajant, 2002).

Proteins of the Bcl-2 family are the major regulators of anoikis (Taddei, Giannoni, Fiaschi, & Chiarugi, 2012). Bcl-2 proteins share conserved Bcl-2 homology (BH)

domains (Scorrano & Korsmeyer, 2003). These proteins can be divided into two groups: anti-apoptotic and pro-apoptotic. The anti-apoptotic Bcl-2 proteins such as Bcl-2 and Bcl-X_L promote cell survival by binding to and inhibiting the function of the pro-apoptotic Bcl-2 proteins. The pro-apoptotic Bcl-2 proteins can be sub-divided into the multi-domain Bcl-2 proteins such as Bax and Bak, and the proteins containing BH3-only domain, such as Bim, Bmf, Bid, Bad etc (Scorrano & Korsmeyer, 2003). Activation of BH3-only Bcl-2 proteins promote apoptosis by triggering translocation of multi-domain Bax or Bak to the mitochondria leading to oligomerization of the latter proteins which cause mitochondrial outer membrane permeabilization (MOMP). MOMP causes the release of cytochrome c into the cytosol and triggers a series of steps leading to caspase-mediated cell death (Figure 3) (Scorrano & Korsmeyer, 2003).

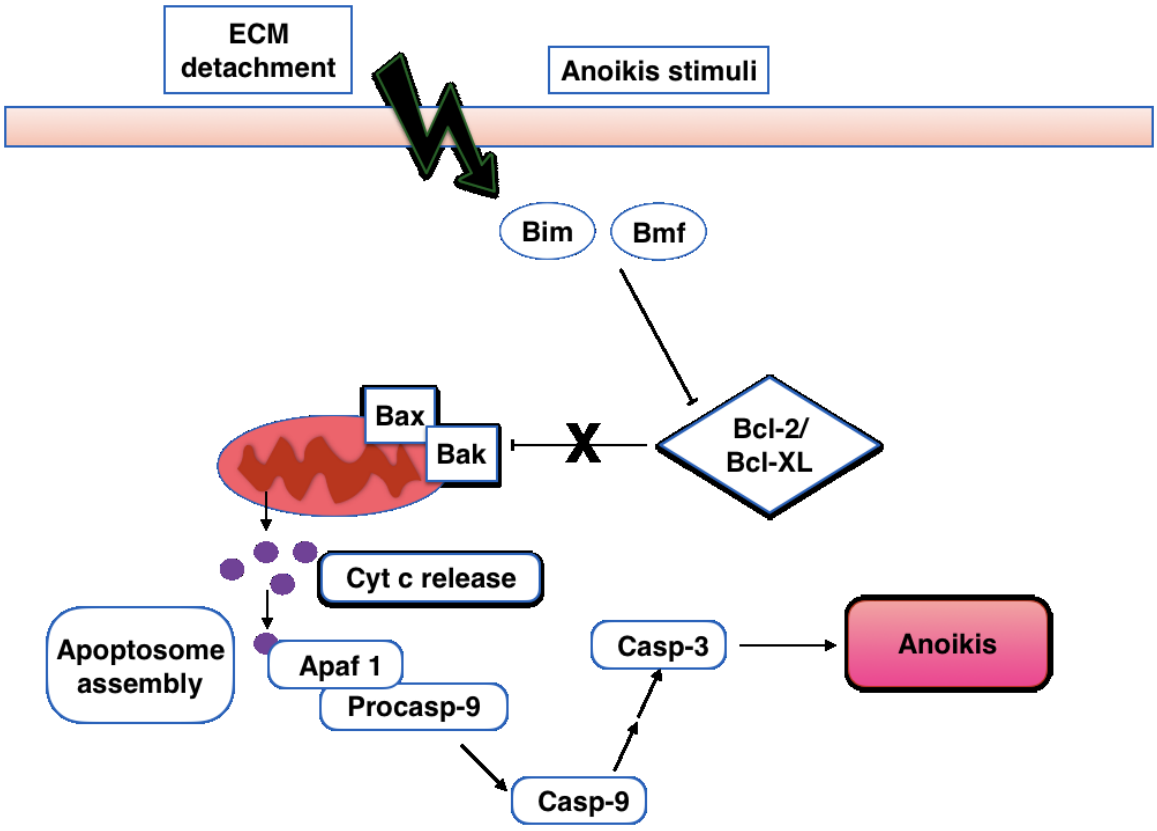

Figure 3. Schematic overview of molecular mechanisms inducing anoikis of breast epithelial cells. Detachment of breast epithelial cells from the ECM acts as a stimulus for activation of caspase-dependent apoptosis, also known as 'anoikis'. The BH3-only Bcl-2 protein such as Bim and Bmf sense the detachment of breast epithelial cells from the ECM and cause neutralization of anti-apoptotic proteins Bcl-2 and Bcl-X_L. Inhibition of Bcl-2 or Bcl-X_L induces oligomerization of pro-apoptotic proteins Bax or Bak. Bax/Bak oligomerization promotes pore formation in the outer mitochondrial membrane (OMM). Mitochondrial outer membrane permeabilization (MOMP) releases cytochrome c into the cytosol where it interacts with the cofactor apoptosis protease activating factor (Apaf-1) to form a protein complex known as 'apoptosome'. Apoptosome recruits pro-caspase-9 leading to its cleavage and activation. Active caspase-9 further cleaves and activates effector pro-caspase-3. Active caspase-3 executes apoptosis by degrading its cellular targets (S. M. Frisch & Screaton, 2001).

1.5.2 The role of Bcl-2 family of proteins in regulating anoikis of breast epithelial cells.

a) The role of Bim in anoikis of breast epithelial cells.

A pro-apoptotic Bcl-2 family member Bim (Bcl-2 interacting mediator of cell death) is sequestered by a complex of cytoskeletal motor proteins (also known as dynein) and actin filaments. Detachment of cells from the ECM causes release of Bim from the motor proteins into the cytosol (Cheng et al., 2001). Bim further translocates to the mitochondria where it neutralizes the pro-survival Bcl-2 protein members, such as Bcl-X_L, and thus triggers apoptosis.

Reginato et al demonstrated that detachment of human breast epithelial cells from the ECM causes an increase in Bim expression which induces anoikis of these cells (M. J. Reginato et al., 2003). Moreover, ablation of Bim by siRNA rescued these cells from anoikis (M. J. Reginato et al., 2003). It was also observed that lack of Bim expression in mice promotes luminal filling of the mammary ducts during mammary gland development (Mailleux et al., 2007). Others have demonstrated that downregulation of Bim expression in mice can delay apoptosis of the mammary gland during involution (Schuler et al., 2016). Thus, detachment-induced upregulation of Bim appears to represent one of the mechanisms of anoikis of breast epithelial cells (Mauricio J. Reginato et al., 2005; M. J. Reginato et al., 2003).

b) The role of Bmf in anoikis of breast epithelial cells.

Bmf (Bcl-2 modifying factor) is normally bound to the myosin V motor proteins, such as the dynein light chain 2. However, following detachment from the ECM Bmf dissociates from this protein and neutralizes the pro-survival Bcl-2 proteins followed by

permeabilization of OMM and release of cytochrome c (Hausmann et al., 2011; Puthalakath et al., 2001). Bmf has been suggested to promote anoikis of breast epithelial cells (Schmelzle et al., 2007). It was observed that enforced downregulation of Bmf alone is sufficient to protect non-malignant breast epithelial cells from anoikis which was further observed to disrupt mammary acinar morphogenesis *in vitro* (Schmelzle et al., 2007). In addition, Bmf was reported to be downregulated by overexpression of pro-survival extracellular-signal regulated kinase (ERK) and Phosphatidyl inositol 3 kinase (PI3K) in detached breast epithelial cells (Y. N. Kim, Koo, Sung, Yun, & Kim, 2012; Schmelzle et al., 2007). Furthermore, it has been demonstrated that Bmf mediates lumen formation within mammary acini of the mouse mammary gland as well as promotes mammary gland involution in mice during the post-lactation period (Schmelzle et al., 2007). Hence, Bmf is another mediator of breast epithelial cell anoikis.

1.5.3 The mechanisms of integrin-dependent inhibition of breast epithelial cell anoikis.

Breast epithelial cells require the integrin cell surface receptors to remain attached to the ECM. Cell-ECM interactions promote survival of these cells (S. M. Frisch & Ruoslahti, 1997). For example, β1 and β4 integrins are commonly known to play a major role in survival of adherent breast epithelial cells (Nisticò, Modugno, Spada, & Bissell, 2014). Integrins were found to block anoikis of breast epithelial cells by activating protein kinases such as focal adhesion kinase (FAK) and the Src protein kinase that are commonly known to activate anti-apoptotic signaling mechanisms (Figure 4) (DeMali, Wennerberg, & Burridge, 2003; Katz & Streuli, 2007).

Failure of integrins to bind to their ECM-ligands have been reported to induce anoikis of breast epithelial cells (Boudreau, Sympson, Werb, & Bissell, 1995; Pullan et al., 1996). For instance, Boudreau and group showed that inhibition of integrin-ECM binding with the help of β1 integrin antibody or forced degradation of the ECM caused by matrix metalloproteinases induces expression of apoptosis inducer interleukin-1β converting enzyme which triggers anoikis of breast epithelial cells (Boudreau et al., 1995; Yuan, Shaham, Ledoux, Ellis, & Horvitz, 1993). Moreover, such inhibition of integrin-ECM binding achieved via β1 integrin antibody was also found to induce expression of anoikis inducer Bmf in breast epithelial cells (Schmelzle et al., 2007). Similarly, it was shown that blocking the ability of integrins to bind to laminins with the help of antibody against laminin also induced apoptosis of the breast epithelial cells (Farrelly et al., 1999). Others have also demonstrated that inhibition of integrin-ECM binding with the help of β1 integrin antibody causes Bax-mediated anoikis of breast and kidney epithelial cells (Andrew P. Gilmore, Metcalfe, Romer, & Streuli, 2000; Pullan et al., 1996; Rytomaa, Lehmann, & Downward, 2000). Hence, it can be concluded that cell-ECM adhesion mediated by integrins is essential to suppress anoikis of various epithelial cell types.

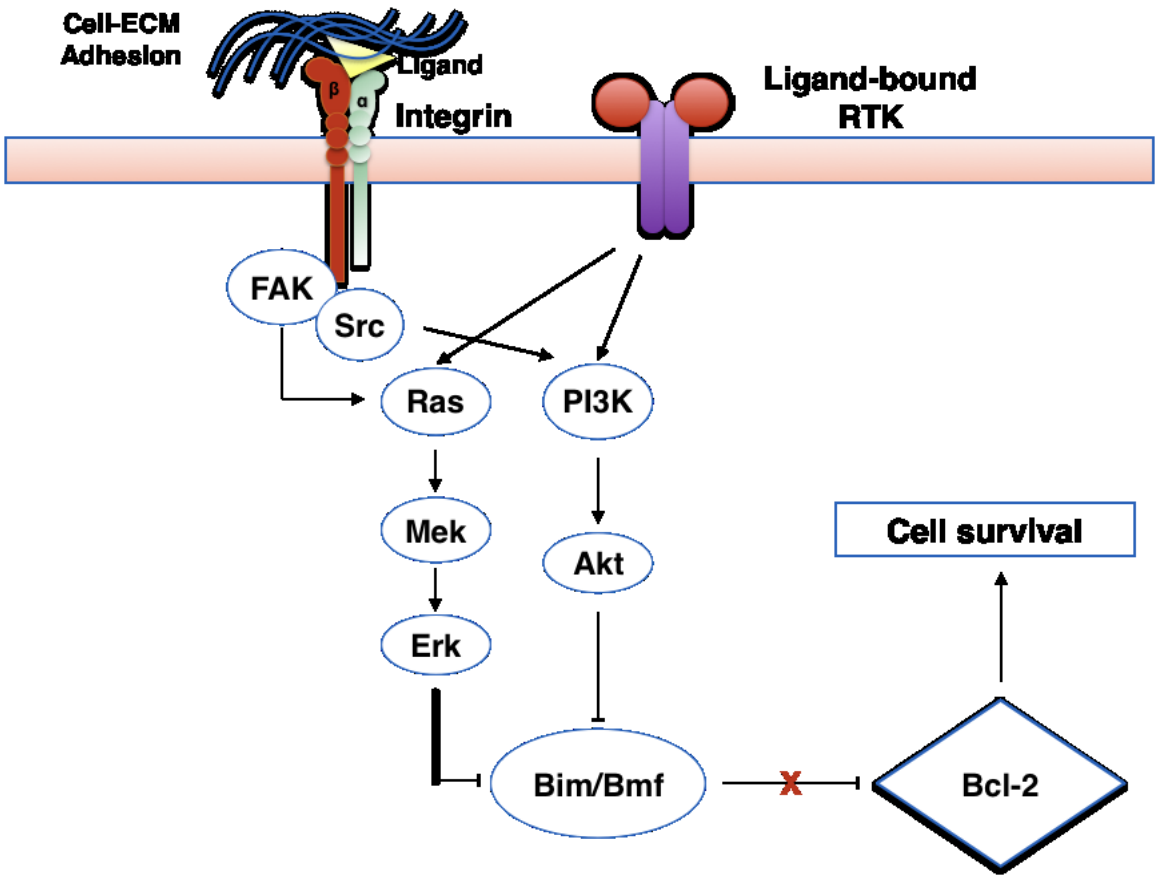

Figure 4. Molecular mechanisms suppressing anoikis after attachment of breast epithelial cells to the ECM. Attachment of integrins to the ECM proteins activates FAK that further activates the Src tyrosine kinases. Src activation leads to further phosphorylation and activation of FAK. Active FAK recruits Grb2 which mediates activation of Ras-Mek-Erk pathway. Activation of Ras causes activation of Mek/Erk kinases. FAK-Src signaling also activates PI3K which further activates Protein Kinase B/Akt kinase. Cell-ECM adhesion also induces activation of receptor tyrosine kinases (RTKs) which function synergistically with integrins to activate common survival signaling pathways. Ras and PI3K pathways together suppress expression of pro-apoptotic Bcl-2 proteins Bim and Bmf which rescues Bcl-2 from neutralization and thus promote breast epithelial cell survival during cell-ECM adhesion (Taddei et al., 2012).

a) The role of focal adhesion kinase in anoikis of breast epithelial cells.

FAK (pp125 focal adhesion kinase) tyrosine kinase is associated with the cytoplasmic tail of integrins (S. M. Frisch, Vuori, Ruoslahti, & Chan-Hui, 1996; Schlaepfer, Hauck, & Sieg, 1999). It was observed that activation of FAK is dependent upon integrin-mediated cell-ECM adhesion (Defilippi et al., 1994). Loss of cell-matrix interaction was demonstrated to inhibit FAK activity in various epithelial cell types such as that of kidney and ovary (Ilic et al., 1998; Lyman, Gilmore, Burridge, Gidwitz, & White, 1997). Activation of integrin was shown to induce tyrosine phosphorylation and activation of FAK which further promotes survival by blocking anoikis of non-malignant epithelial cells (Steven M. Frisch et al., 1996). Frisch et al., showed that expression of constitutively active FAK rescued kidney epithelial cells from anoikis (Steven M. Frisch et al., 1996). FAK-mediated activation of PI3K and the Src kinase pathways were suggested to promote survival of detached breast epithelial cells (Andrew P. Gilmore et al., 2000). Furthermore, it was demonstrated that inhibition of FAK activity induces translocation of pro-apoptotic Bax to the mitochondria in detached mouse mammary epithelial cells resulting into apoptotic cell death (Andrew P. Gilmore et al., 2000). Thus, FAK plays an essential role in mediating adhesion dependent survival of breast epithelial cells.

b) The role of mitogen-activated protein kinase kinase in anoikis of breast epithelial cells.

Mitogen activated protein kinase kinase or Mek is a dual specificity kinase that can act as both tyrosine and serine/threonine kinase. It stimulates pro-survival

mechanisms by inducing activation of extracellular-signal regulated kinases (ERK) also known as the mitogen-activated protein kinase (MAPK) (Dhillon, Hagan, Rath, & Kolch, 2007). Integrin-mediated activation of FAK was found to promote activation of Erk kinase in adhered fibroblast cells (Schlaepfer, Hanks, Hunter, & van der Geer, 1994) (Moro et al., 1998). In line with these observations, Mauricio and group observed that integrins and epidermal growth factor receptor (EGFR) co-ordinate with each other to block anoikis of breast epithelial cells (M. J. Reginato et al., 2003). They found that inhibition of β1 integrin attachment to the ECM results in loss of epidermal growth factor receptor (EGFR) expression in detached breast epithelial cells (M. J. Reginato et al., 2003). Downregulation of EGFR was found to block activation of Mek-Erk pathway and thus induce anoikis of breast epithelial cells due to upregulation of Bim expression (M. J. Reginato et al., 2003). Furthermore, constitutive activation of Mek kinase was found to suppress Bim expression which rescued breast epithelial cells from anoikis (M. J. Reginato et al., 2003). Hence, active Mek kinases are required for adhesion dependent survival of breast epithelial cells.

c) *The role of Phosphatidyl inositol 3-kinase and protein kinase B in anoikis of breast epithelial cells.*

One of the signalling molecules activated by integrins in response to cell-ECM adhesion is PI3K or Protein Kinase B/Akt (H.-C. Chen, Appeddu, Isoda, & Guan, 1996; H. Xia, Nho, Kahm, Kleidon, & Henke, 2004). Loss of PI3K activity due to disruption cell-ECM adhesion has been well-documented to cause anoikis of various epithelial cell types (Khwaja, Rodriguez-Viciana, Wennstrom, Warne, & Downward, 1997; King, Mattaliano, Chan, Tsichlis, & Brugge, 1997; T. H. Lin, Chen, Howe, & Juliano, 1997).

Integrins-induced activation of FAK via phosphorylation of specific tyrosine residues creates docking sites for p85 regulatory subunit of PI3K upstream of Akt (H.-C. Chen et al., 1996). Loss of integrin engagement with ECM was found to cause inhibition of FAK activity followed by suppression of PI3K in detached breast epithelial cells (Attwell, Roskelley, & Dedhar, 2000; Andrew P. Gilmore et al., 2000). Furthermore, lack of PI3K activity was observed to induce Bax-dependent apoptosis of the indicated cells (Andrew P. Gilmore et al., 2000; Menard, Jovanovski, & Mattingly, 2005). It was also observed that integrin-laminin binding induced insulin receptor-mediated activation of PI3K/Akt to promote cell survival (Farrelly et al., 1999). Ligand-induced activation of insulin receptor causes recruitment of adaptor protein insulin receptor substrate-1 (IRS-1) (X. J. Sun et al., 1992). Formation of insulin receptor-IRS-1 complex triggers tyrosine phosphorylation on IRS-1 followed by recruitment and activation of PI3K which eventually activates Protein Kinase B/Akt kinase (Myers et al., 1994; X. J. Sun et al., 1992). Loss of cell-ECM adhesion inhibits recruitment of IRS-1 to the insulin receptor and thus causes apoptotic cell death due to inhibition of PI3K activity (Farrelly et al., 1999). Hence, PI3K is an essential mediator of adhesion dependent survival of breast epithelial cells which prevents anoikis.

d) The role of p38alpha mitogen-activated protein kinase in anoikis of breast epithelial cells.

p38α MAPK is a stress activated protein kinase involved in inducing anoikis of epithelial cells (Vachon et al., 2002). p38 MAPK responds to a wide range of stress stimuli such as heat shock, ultraviolet irradiation and osmotic shock and plays an important role in cell apoptosis (Owens et al., 2009). In breast epithelial cells, p38alpha

activity was found to induce anoikis of the cells by promoting Bax induced MOMP to release cytochrome c and commit the cells towards apoptosis (Owens et al., 2009). p38alpha was also found to stimulate expression of death promoting extra-long isoform of Bim (BimEL) in detached breast epithelial cells (Wen et al., 2011). It was observed that p38alpha-induced BimEL expression resulted in formation of lumen within a mammary duct (Wen et al., 2011). Furthermore, pharmacological inhibition of p38α increased cell survival and luminal filling of mammary duct which facilitated mammary tumourigenesis in mice expressing ErbB2 oncoprotein (Wen et al., 2011).

Apart from breast epithelial cells, others have associated expression of p38 isoforms with downregulation of FAK and PI 3K activity in intestinal epithelial cells which eventually contributes to anoikis (Vachon et al., 2002). In addition, it was observed that p38α induced anoikis of intestinal epithelial cells by increasing the expression of Fas ligand and subsequent activation of the extrinsic apoptotic pathway (Rosen, Shi, Calabretta, & Filmus, 2002). Hence, p38α MAPK promotes anoikis of different epithelial cell types by downregulating the pro-survival factors.

e) The role of Jun-N terminal kinases in anoikis of breast epithelial cells.

Jun-N terminal kinases (JNK) are stress activated protein kinase that respond to stress-induced stimuli including cell detachment from the ECM. It has been demonstrated to mediate anoikis of kidney epithelial cells (S. M. Frisch, Vuori, Kelaita, & Sicks, 1996; Girnius & Davis, 2017). JNK activity was also observed to promote lumen formation within a mammary acinus *in vitro* (McNally et al., 2011). It was further observed that gene silencing of JNKs resulted in luminal filling of the mammary ducts *in vivo*

(McNally et al., 2011). Furthermore, it was shown that mice deficient of JNK displayed dependence on JNK activity for promoting lumen formation and apoptosis in the terminal end buds of the mouse mammary gland (a process typically known to be executed by anoikis-inducing mechanisms) (Girnius & Davis, 2017). Thus, JNK is suggested to play an important role in the induction of anoikis of breast epithelial cells.

1.6 Non-apoptotic mechanisms of breast epithelial cell death after detachment from the ECM.

Non-apoptotic mechanisms of cell death occur independent of classical apoptotic mechanisms or when apoptosis mechanisms have been blocked (Debnath et al., 2002; Tait, Ichim, & Green, 2014). It has been observed that detached human primary breast epithelial cells harbouring inactive apoptotic machinery undergo cell death through induction of non-apoptotic processes such as entosis, necrosis or lysosomal-cathepsin induced cell death (Ishikawa, Ushida, Mori, & Shibanuma, 2015).

1.6.1 The role of Entosis in detachment-induced death of breast epithelial cells.

Entosis is a Greek word which means ‘inside’ or ‘into’. It is a process where one cell invades the other to either survive within the host cell or to be degraded by the host lysosomes in order to provide metabolic nutrients for the survival of the detached host cell (Buchheit, Rayavarapu, & Schafer, 2012). Overholtzer and colleagues discovered in this regard that detached breast epithelial cells can invade one another and undergo degradation in the lysosomes (Overholtzer et al., 2007). It was further observed that entosis of detached breast epithelial cells occurred in a caspase-independent manner (Overholtzer et al., 2007). Overexpression of anti-apoptotic protein Bcl-2 or treatment

with caspase inhibitors did not block cell internalization of detached breast epithelial cells (Overholtzer et al., 2007). Furthermore, detachment-induced death of internalized breast epithelial cells was observed to majorly occur via lysosomal degradation (see section 1.6.4) (Overholtzer et al., 2007). It was demonstrated that overexpression of Bcl-2 in detached breast epithelial cells partially rescued the internalized cells from lysosomal cell death (see section 1.6.4) (Overholtzer et al., 2007). However, simultaneous treatment of Bcl-2-overexpressing breast epithelial cells with lysosomal inhibitor significantly rescued the cells from detachment-induced cell death (Overholtzer et al., 2007). Therefore, these data suggest that cells undergoing entosis during matrix detachment majorly undergo lysosomal cell-death, however, a defective lysosomal cell death pathway can induce apoptotic cell death in detached breast epithelial cells (Overholtzer et al., 2007).

1.6.2 The role of necrosis mechanisms in detachment-induced death of breast epithelial cells.

Necrosis is a form of unregulated cell death caused due to an accident (Tsujimoto, 2012). However, necrosis can also be driven by a molecular program. This form of necrosis is called ‘necroptosis’ or ‘programmed necrosis’ (Cho et al., 2009; He et al., 2009) which involves formation of a multiprotein complex called ‘necrosome’ in the absence of caspase-8 activity. A necrosome includes receptor interacting protein kinases (RIPK) 1 and 3 and mixed-lineage kinase domain-like protein (MLKL) (Chan, Luz, & Moriwaki, 2014; Pasparakis & Vandenabeele, 2015).

Whether or not programmed necrosis plays a role in promoting detachment-induced death of breast epithelial cells has not been well understood. However, some

evidence suggest necrosis to be an alternative mode of detachment-induced death of breast epithelial cells but in a certain cellular context. For instance, detached primary human breast epithelial cells harboring defective apoptosis machinery were found to undergo necrotic cell death (Ishikawa et al., 2015). These cells when detached from the ECM were observed to significantly stain positive for propidium iodide (a hallmark for necrosis (Kabakov, Kudryavtsev, & Gabai, 2011)) rather than Annexin-V (a hallmark for apoptosis (Kabakov et al., 2011)). It was also noticed that these cells underwent detachment-induced entosis which was further followed by necrotic cell death (Ishikawa et al., 2015). Moreover, overexpression of anti-apoptotic proteins such as ErbB2, PI3K, Src kinase, Mek or Bcl-X_L could not rescue these cells from death after detachment from the ECM (Ishikawa et al., 2015). This suggests that detachment-induced necrotic death of breast epithelial cells may depend upon the functional status of the apoptotic pathways in those cells (Ishikawa et al., 2015).

1.6.3 Generation of *reactive oxygen species*

Reactive Oxygen Species (ROS) are generated in cells by electron reduction of O_2 in the process of production of water molecules (Nathan & Ding, 2010). Some of the examples for ROS are superoxide anion radical, hydrogen peroxide and hydroxyl radical (Nathan & Ding, 2010). The major sources of total pool of ROS generated in the detached breast epithelial cells are the mitochondria and the endoplasmic reticulum (ER) (Avivar-Valderas et al., 2011; Zeeshan, Lee, Kim, & Chae, 2016).

The mitochondrial ROS are generated in the electron transport chain during the process of oxidative phosphorylation in the inner mitochondrial membrane (Chaube &

Werstuck, 2016; Nathan & Ding, 2010). Leakage of electrons from the mitochondrial complexes I and III leads to reduction of oxygen to superoxide anion followed by subsequent dismutation to hydrogen peroxide catalyzed by enzymes superoxide dismutase 1 and 2 located in the intermitochondrial membrane space (Chaube & Werstuck, 2016). In ER, oxidative protein folding mediated by proteins such as ERO-1 (ER oxidoreductin-1), protein disulfide isomerase (PDI) and reduced glutathione (GSH) result in generation of ROS (Zeeshan et al., 2016). During protein folding, ERO-1 is oxidized by molecular oxygen which then acts as an oxidant for PDI (Zeeshan et al., 2016). PDI then induces disulfide bonds formation for folding of proteins (Zeeshan et al., 2016). This process of oxidative protein folding leads to generation of ROS and oxidized glutathione (GSSG) that adds to the oxidative stress of the cells (Zeeshan et al., 2016). Accumulation of unfolded proteins can induce the unfolded protein response (UPR) to compensate for the defective protein folding machinery resulting into added production of ROS (Zeeshan et al., 2016). Furthermore, the release of calcium ions from the ER during stress conditions result in the increased uptake of calcium ions by the mitochondria leading to mitochondrial dysfunction and thus can trigger cell death (Tu & Weissman, 2004). Neutralization of excessive ROS involves production of GSH in the cells (Mailloux, Ayre, & Christian, 2016). GSH quenches hydrogen peroxide with the help of enzyme glutathione peroxidase and thus generate GSSG which is again reduced by NADPH (nicotinamide adenine dinucleotide phosphate) with the help of an enzyme glutathione reductase (Mailloux et al., 2016).

Adhered breast epithelial cells promote glucose oxidation via mitochondrial tricarboxylic acid cycle to generate ATP (Schafer et al., 2009). However, detachment of

breast epithelial cells leads to reduction in glucose uptake, generation of reactive oxygen species (ROS) followed by depletion of ATP levels in a caspase-independent manner (Schafer et al., 2009). Reduced glucose levels inhibit production of antioxidants such as NADPH through the pentose phosphate pathway (PPP) (Schafer et al., 2009). This in turn causes a significant increase in the levels of ROS which further inhibits fatty acid oxidation (FAO) pathway and thus suppress ATP generation (Figure 5) (Schafer et al., 2009). ROS has been previously implicated in stimulating cytochrome c release from the mitochondria to initiate apoptosis (Orrenius, Gogvadze, & Zhivotovsky, 2007). However, Schafer agroup demonstrated that during lumen formation in the mammary acini in tissue culture, ROS species get accumulated in the centrally located detached breast epithelial cells causing ATP depletion and detachment-induced cell death (Schafer et al., 2009). They found that overexpression of anti-apoptotic protein Bcl-2 could not promote long-term anchorage-independent survival of these cells (Schafer et al., 2009). Therefore, it can be concluded that excessive amount of ROS generated in detached breast epithelial cells can cause detachment-induced cell death in a caspase-independent manner.

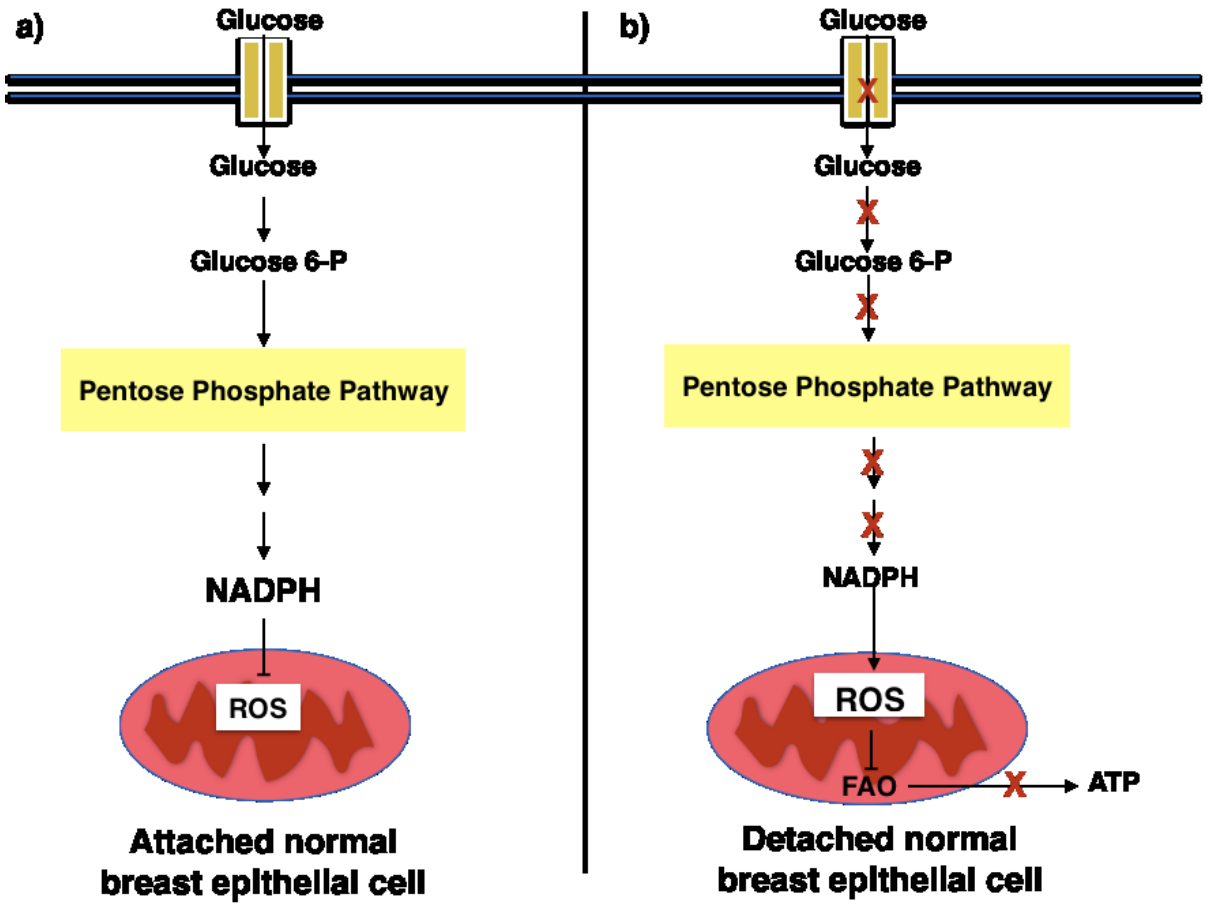

Figure 5. Schematic representation of ROS regulation by cell-ECM adhesion. (a) In attached normal cells, glucose is transported into the cells through glucose transporter and is converted into glucose-6-phosphate by hexokinase. Glucose-6-phosphate is metabolized through pentose phosphate pathway to generate a reducing agent NADPH through a series of enzymatic reactions. NADPH neutralizes ROS generated by the mitochondria and prevents ROS-mediated degradation of the cellular substrates. (b) When the cells detach from the ECM, glucose transport into the cell cytosol is abrogated due to downregulation of glucose transporters. Reduced uptake of glucose blocks glucose metabolism via PPP leading to reduced production of NADPH. Low levels of NADPH increase ROS levels which blocks ATP generation by fatty acid oxidation (FAO) in the mitochondria (Schafer et al., 2009).

1.6.4 The role of lysosomal cathepsin-dependent mechanisms of death of detached breast epithelial cells.

Lysosomes, also known as “suicide bags”, are single membrane-bound vesicles that contain hydrolases required to degrade biomolecules such as lipids, nucleic acids, carbohydrates and protein molecules within the cells in an acidic environment (Cuervo & Dice, 1997; Duve, 1983). Lysosomal degradation of molecules can occur via endocytosis, autophagy and phagocytosis (Turk & Turk, 2009).

In the endocytic pathway, clathrin-coated endocytic vesicles (vesicles that contain extracellular molecules) bud off from the plasma membrane and form the early endosome. The early endosome gradually matures into the late endosome. The acidic pH of the late endosomes facilitates the degradative activity of the lysosomal enzymes delivered by the fusion of vesicles generated by the Golgi apparatus (Luzio, Pryor, & Bright, 2007).

In autophagy (also known as “self-eating”), a double-membrane vesicle also known as “autophagosomes” is formed which is involved in engulfing intracellular organelles, proteins and other macromolecules and deliver it to the lysosomes by formation of “autolysosomes” (see section 1.7.4) (L. Yu, Chen, & Tooze, 2017). Cytosolic chaperones such as heat shock proteins Hsc70 and Hsp90 deliver specific proteins to the lysosomal surface by binding the target proteins to the lysosome-associated membrane-protein 2A (LAMP-2A) followed by internalization and degradation of the target protein within lysosome lumen (Kaushik & Cuervo, 2018).

Phagocytosis is a form of endocytosis that is clathrin-independent and involves lysosomal degradation of endocytosed foreign particles such as dust, cell debris and apoptotic cells in specialized cells such as macrophages (Bohdanowicz & Grinstein, 2013).

Out of the many hydrolases found in lysosomes, proteases such as cathepsins play an important role in protein degradation. Cathepsins are the lysosomal proteases that are released into the cell cytosol after lysosomal membrane permeabilization (LMP) caused by diverse stimuli, such as the generation of ROS (Terman, Kurz, Gustafsson, & Brunk, 2006) or cell treatment with lysosomal membrane destabilizing reagents (W. Li et al., 2000). Cathepsins can induce cell death in a caspase-dependent or -independent manner (P. Boya & Kroemer, 2008). Partial disruption of lysosomal membrane was observed to typically induce apoptosis (P. Boya & Kroemer, 2008; KÅGedal, Zhao, Svensson, & Brunk, 2001). LMP was suggested to be caused by translocation of pro-apoptotic Bcl-2 protein Bax to the lysosomes thereby releasing lysosomal proteases in the cytosol (Kågedal et al., 2005). The lysosomal proteases induce mitochondrial membrane permeabilization (MOMP) by activating BH3-only protein Bid (P. Boya & Kroemer, 2008). MOMP releases cytochrome c which triggers caspase-dependent cell death (Patricia Boya et al., 2003; P. Boya & Kroemer, 2008). However, complete disruption of lysosomal membrane results in accumulation of lysosomal proteases in the cell cytosol at high concentrations leading to caspase independent cell death (KÅGedal et al., 2001). Overholtzer and colleagues found that breast epithelial cells undergo lysosomal cathepsin-dependent cell death after detachment-induced Entosis (Overholtzer et al., 2007). Others also demonstrated that the release of Cathepsins from the lysosomes

contributes to mammary gland regression during involution, a process that is thought to be driven at least in part by destruction of the ECM to which breast epithelial cells are attached (Watson & Kreuzaler, 2011).

Hence, detachment-induced death of breast epithelial cells occur in both apoptosis-dependent and independent manner. Non-apoptotic cell death mechanisms are alternative routes acquired by the cells to execute detachment-induced cell death in the absence of apoptotic mechanisms. Based on these evidences, it can be inferred that inhibition of both apoptotic and non-apoptotic mechanisms of cell death may be essential for long-term survival of detached cancer cells. Therefore, therapeutic approaches aimed at inhibiting survival pathways that suppress both apoptotic and non-apoptotic cell death mechanisms in detached breast cancer cells may prove beneficial in effective treatment of cancer.

1.7 Molecular mechanisms of anoikis resistance of breast cancer cells.

1.7.1 The role of oncoproteins and tumour suppressor proteins in anoikis resistance of breast cancer cells.

Expression of oncoproteins, such as EGFR, ErbB2, Raf, Src and PI3K/Akt (Derouet et al., 2007; I. A. Khan et al., 2016; Marani et al., 2004; M. J. Reginato et al., 2003) or loss of tumour suppressor proteins, such as p53 (apoptotic factor) and phosphatase and tensin homolog (PTEN, an antagonist of PI3K) (B. S. Tan et al., 2015; Vitolo et al., 2009) was found to induce constitutive activation of signalling pathways that block anoikis of various types of cancer cells, such as those of breast, colon, lung, prostate and pancreas.

Reginato et. al., observed that overexpression of EGFR rescued detached breast epithelial cells from anoikis by activating the MAPK pathway followed by downregulation of the pro-apoptotic factor Bim (M. J. Reginato et al., 2003). Similarly, increased Src tyrosine kinase activity was shown to promote anoikis resistance of ErbB2-overproducing breast epithelial cells by also promoting suppression of Bim expression via constitutive activation of Mek-Erk pathway (Haenssen et al., 2010). ErbB2 overexpression was also found to stabilize EGFR expression in an Erk-dependent manner and blocked anoikis of breast epithelial cells by suppressing Bim expression (Grassian, Schafer, & Brugge, 2011). Apart from ErbB2-mediated activation of Mek-Erk pathway, constitutive activation of Raf kinase (a component of Ras-MAPK pathway) was also shown to promote anoikis resistance of breast epithelial cells by downregulating Bim expression (Gall et al., 2000; Marani et al., 2004).

Active mutations of gene encoding PI3K is known to occur in 26% of breast carcinoma (Saal et al., 2005). Overexpression of PI3K was shown to promote anoikis resistance of breast epithelial cells via several signaling pathways that inhibit apoptosis-inducing mechanisms (Attwell et al., 2000; Y. N. Kim et al., 2012; Paolo Paoli, Elisa Giannoni, & Paola Chiarugi, 2013). Schulze et al., demonstrated that constitutive activation of Raf kinase in detached breast epithelial cells promotes anoikis resistance of the cells in a PI3K dependent manner (Gall et al., 2000). Furthermore, constitutive activation of Akt was found to suppress expression of pro-apoptotic factors Bim and Bmf (BH3-only Bcl-2 protein) in detached breast epithelial cells which promoted their survival (Haenssen et al., 2010; Schmelzle et al., 2007). In addition to overexpression or constitutive activation of PI3K/Akt pathway, loss of tumour suppressor genes such as

PTEN and p53 were also shown to promote anoikis resistance of breast cancer cells by causing uncontrolled activation of PI3K pathway (Bertheau et al., 2013; Vitolo et al., 2009).

The ability of Akt to block anoikis is not unique to breast epithelial cells. For instance, Akt was found to mediate expression of transcription factor nuclear factor κB (NF-κB) that induced upregulation of anti-apoptotic protein Bcl-2 and an inhibitor of apoptosis-1 (IAP-1) protein to promote anoikis resistance of intestinal epithelial cells (Toruner et al., 2006). In addition, PI3K was also demonstrated to mediate downregulation of a pro-apoptotic protein Bak which induced anoikis resistance of ras-transformed intestinal epithelial cells (Liu et al., 2006).

Hence, expression of oncoproteins and loss of tumour suppressor genes play an important role as inhibitors of anoikis of epithelial cells of the breast and other organs.

1.7.2 The role of hypoxia in anoikis resistance of breast cancer cells.

Tumour cells are often hypoxic due to the disorganized nature of the tumour vasculature which normally supplies the cells with oxygen. Hypoxia is well-known to induce expression of the transcription factor hypoxia inducible factor-1 (HIF-1). HIF1 was in turn found to block anoikis of breast epithelial cells by suppressing Bim expression through activation of the Mek-Erk signalling pathway. Moreover, HIF1-dependent mechanism was found to inhibit luminal clearance during mammary acinar morphogenesis *in vitro* (K. A. Whelan et al., 2010). Finally, it was observed that the tumour cells derived from transgenic mice harboring ErbB2 oncoprotein expressed higher levels of HIF-1 compared to normal epithelial cells (Kelly A. Whelan et al., 2013).

Hence, in addition to the anti-anoikis pathways triggered by the activation of oncogenes or loss of tumour suppressor genes, hypoxia (to which breast tumour cells are often subjected *in vivo*) can contribute to the inhibition of anoikis of breast cancer cells.

1.7.3 The role of cancer cell metabolism and oxidative stress in anoikis resistance of breast cancer cells.

Detachment of breast epithelial cells was shown to induce activation of pyruvate dehydrogenase kinase (PDK4) which prevents mitochondrial oxidation of glucose resulting into reduced production of ROS and increased survival (Kamarajugadda et al., 2012). PDK is an inhibitor of enzyme pyruvate dehydrogenase (PDH) which is required to promote glucose oxidation (Hawk & Schafer, 2018). PDKs are overexpressed in detached cancer cells for promoting anoikis resistance and prolonged cell survival during nutrient starvation (Kamarajugadda et al., 2012).

Schafer and group found that the breast epithelial cells die due detachment-induced upregulation of ROS followed by suppression of FAO-mediated ATP generation. They observed that treatment of breast epithelial cells with antioxidants rescued the cells from detachment-induced cell death and promoted luminal filling *in vitro* (Schafer et al., 2009).

It was also observed that overexpression of oncoprotein such as ErbB2 promoted survival of detached breast epithelial cells by PI3K-mediated increase in glucose uptake (Schafer et al., 2009). Glucose can further be metabolized via pentose phosphate pathway (PPP) to generate a reducing agent nicotinamide adenine dinucleotide phosphate (NADPH) (Cairns, Harris, & Mak, 2011). NADPH was further observed to

neutralize ROS which rescued cellular ATP levels and promoted survival of breast epithelial cells when detached from the ECM (Schafer et al., 2009). However, the ability of oncoproteins such as Bcl-2 and ErbB2 to promote long-term anchorage-independent survival of breast epithelial cells was found to be insufficient and thus required further reduction of excessive amounts of ROS (Schafer et al., 2009). It was observed that treatment of detached breast cancer cells overexpressing ErbB2 or Bcl-2 (that are known to suppress anoikis (Debnath et al., 2002; M. J. Reginato et al., 2003)) with antioxidants neutralized excessive ROS and promoted prolonged anchorage-independent survival of breast cancer cells (Figure 6) (Schafer et al., 2009). Hence, based on this data it can be suggested that induction of anoikis resistance along with concomitant inhibition of ROS-mediated cell death can promote long-term survival of detached breast cancer cells.

Other mechanisms involved in neutralization of ROS during breast epithelial cell detachment involves activation of liver kinase B1 (LKB1)- AMP-activated protein kinase (AMPK) pathway (Hawk & Schafer, 2018). During energy stress, AMPK is activated by LKB1 and Ca2+/calmodulin dependent kinase kinase beta (Sundararaman, Amirtham, & Rangarajan, 2016). Active AMPK inhibits consumption of NADPH during fatty acid oxidation by stabilizing NADPH levels in the cells and thus reduce ROS levels in the cells (Jeon, Chandel, & Hay, 2012). Furthermore, it has been observed that overexpression of superoxide dismutase 2 (SOD2) in detached breast epithelial cells promoted cell survival by limiting the detrimental effects of ROS in the cells and thus prolong cell survival.

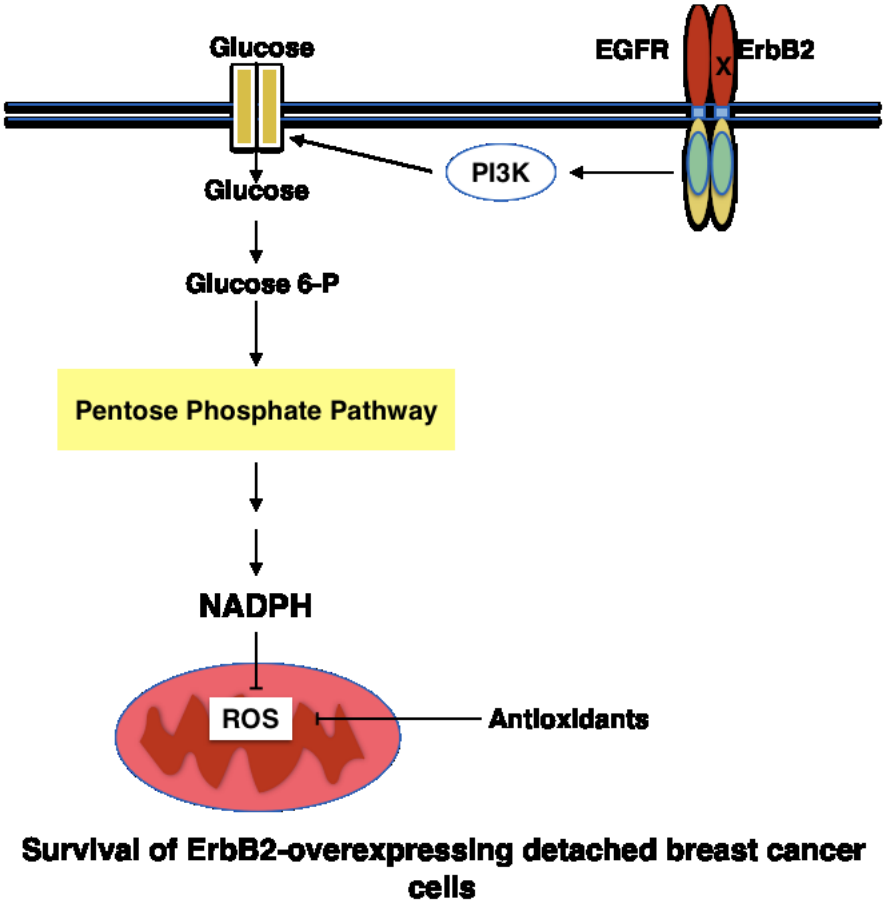

Figure 6. Role of cancer cell metabolism and oxidative stress in resistance of breast cancer cells to detachment-induced cell death. Oncogenic transformation of breast epithelial cells due to ErbB2-overexpression leads to constitutive activation of PI3K pathway. PI3K increases uptake of glucose into the cytosol via upregulation of glucose transporter. Imported glucose can enter the pentose phosphate pathway (PPP). PPP mediated generation of NADPH neutralizes ROS in the cell cytosol. Antioxidant treatment further suppresses ROS and prolongs survival of detached breast cancer cells (Schafer et al., 2009).

1.7.4 The role of autophagy in anoikis resistance of breast cancer cells.

Autophagy (derived from the Greek meaning 'eating of self') has been suggested to promote long-term survival of anoikis resistant breast cancer cells during cancer progression (Fung, Lock, Gao, Salas, & Debnath, 2008). It is a process that initiates with the formation of ER-derived double membrane vesicle also known as pre-autophagosome due to activation of class III phosphatidylinositol 3-kinase (PI3K) complex that synthesizes phosphatidylinositol 3-phosphate lipids required for the formation of the vesicle membrane (Feng, He, Yao, & Klionsky, 2013). At the pre-autophagosome stage, ATG-related proteins ATG12-5-16L1 complex recruits microtubule associated protein 1 light chain 3 (LC3I) to the membrane and lipidates the latter protein into LC3II by binding LC3I to phosphatidyl ethanolamine (Mizushima, Yoshimori, & Ohsumi, 2011). This step is required for the maturation of autophagosomes (Mizushima et al., 2011). LC3II then promotes autophagosome and lysosome fusion resulting into lysosomal degradation of intracellular contents delivered by the autophagosomes (Mizushima et al., 2011).

It was observed that inhibition of autophagy suppressed clonogenic survival of detached breast epithelial cells overexpressing anti-apoptotic protein Bcl-2 (Fung et al., 2008). In addition, activation of endoplasmic reticulum kinase PERK (known to inhibit protein translation) due to detachment-induced generation of ROS was found to promote autophagy in breast epithelial cells which enhanced cell survival and hence delayed anoikis of the indicated cells (Avivar-Valderas et al., 2011). Furthermore, detection of active PERK and concomitant increase in autophagy in breast tumour cells developed in the ducts of the mammary gland indicated an important role for autophagy in protecting

cancer cells during various steps of cancer progression such as dissemination and metastasis (Avivar-Valderas et al., 2011).

1.7.5 The role of microRNAs in anoikis resistance of breast cancer cells.

MicroRNAs or miRNAs are short RNA molecules that are complementary to the target mRNA sequences to which they can bind and trigger their silencing through recruitment of miRNA-induced silencing complex (miRISC). miRISC was suggested to either block translation or cause degradation of respective mRNAs (Fung et al., 2008; Jansson & Lund, 2012). miRNA family members are known to function as both promoters or inhibitors of anoikis sensitivity in cancer cells (Malagobadan & Nagoor, 2015). For instance, miR-181a-5p expression was found to be upregulated in detached breast epithelial cells which inhibited autophagy-induced survival of the indicated cells (Fung et al., 2008). However, expression of miR-181a in detached breast cancer cells was shown to promote anoikis resistance of breast cancer cells by causing downregulation of Bim expression (M. A. Taylor, Sossey-Alaoui, Thompson, Danielpour, & Schiemann, 2013). Similarly, miR200b has been demonstrated to promote anoikis susceptibility of breast cancer cells by regulating the Erk signalling pathway (Fung et al., 2008) whereas miR200a was shown to promote anoikis resistance of breast cancer cells by inducing downregulation of tumour suppressor protein YAP1 (Yes associated protein). Inhibition of YAP1 was in turn found to trigger activation of the Akt signalling pathway which is well known to block anoikis of breast cancer cells (S.-J. Yu et al., 2013).

1.8 The role of breast tumour cell anoikis resistance in breast cancer progression.

The ability of cancer cell to resist anoikis is thought to be critical for tumour initiation, invasion and metastasis (Y. N. Kim et al., 2012; Taddei et al., 2012). Unlike normal epithelial cells that typically grow attached to the ECM, carcinoma cells tend to form three-dimensional disorganized multicellular masses in which many cells are no longer attached to the ECM (Debnath & Brugge, 2005). Moreover, tumours often produce the basement membrane-degrading enzymes called matrix metalloproteases that destroy the ECM and thus promote tumour invasion into the adjacent tissues (Dalberg, Eriksson, Enberg, Kjellman, & Backdahl, 2000; Ljubimov et al., 1992). During advanced stages of carcinoma, cancer cells enter into the blood circulation wherein they remain detached from the ECM and yet remain viable. Eventually, the circulating cancer cells disseminate through the body and form metastases (Figure 7) (Berezovskaya et al., 2005; Douma et al., 2004). Thus, anoikis resistance of malignant cells is likely essential for all of the indicated events (Y. N. Kim et al., 2012).

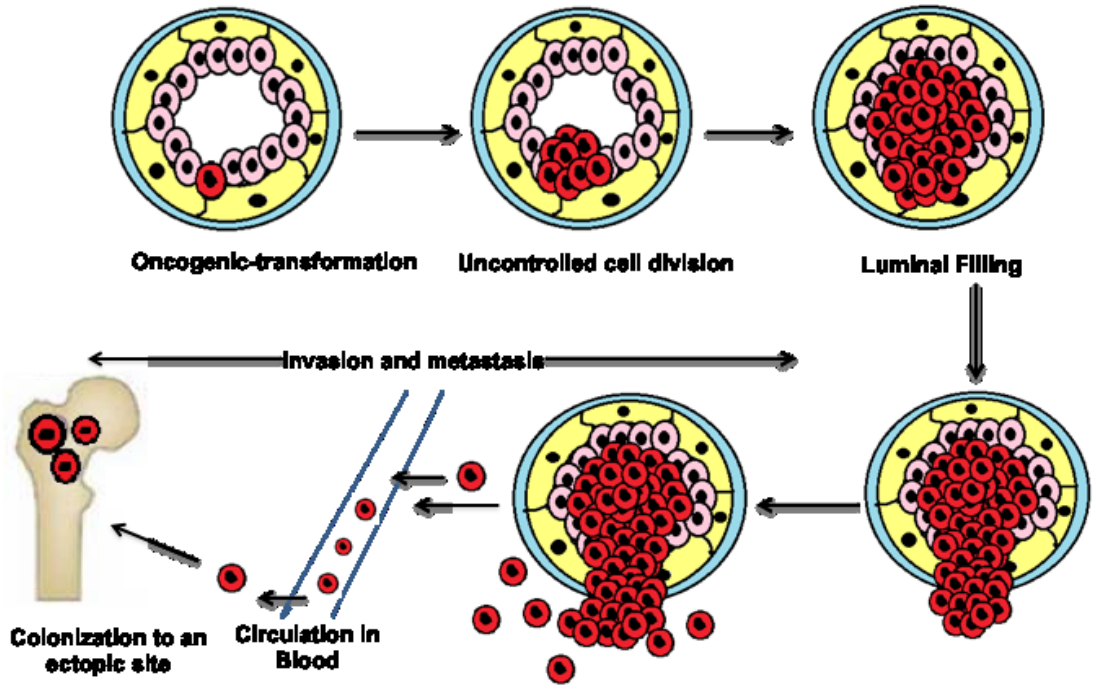

Figure 7. Schematic representation of the physiological significance of anoikis resistance for breast cancer progression. Expression of oncoproteins in breast epithelial cells results in uncontrolled cellular survival and growth. As a consequence, the cell masses fill the mammary duct. Cancerous cells within tumours are likely deprived of proper contact then destroy the surrounding ECM, invade the adjacent tissues and enter into the blood circulation without being attached to the ECM. These circulating cancer cells while being detached and viable, form metastases (P. Paoli, E. Giannoni, & P. Chiarugi, 2013; Scully, Bay, Yip, & Yu, 2012).

1.8.1 Breast cancer cell anoikis resistance as a hallmark of epithelial-mesenchymal transition.

Anoikis resistance is considered as a critical feature of epithelial-mesenchymal transition. Tumour initiation is thought to be preceded by a process called epithelial-mesenchymal transition (EMT), a process that defines conversion of the normal epithelial cells into the malignant ones (S. M. Frisch, Schaller, & Cieply, 2013). Cells undergoing

EMT lose epithelial cell specific properties such as a well-defined cell-to-cell adhesion responsible for the “cobblestone-like” morphology of epithelial layers, apical-basal polarity and cell-ECM adhesion (Wheelock, Shintani, Maeda, Fukumoto, & Johnson, 2008). Eventually, these cells acquire the features of the mesenchymal phenotype characterized by loss of E-cadherin, increased expression of N-cadherin and fibronectin, increased migratory behaviour, invasiveness and resistance to apoptosis (Kalluri & Weinberg, 2009). Lack of dependence on adhesion to the ECM for cell survival is one of the proposed steps of the EMT (Mani et al., 2008).

1.8.2 Breast cancer cells anoikis resistance as a critical feature of cancer stem cells.

Another critical feature of breast cancer cells required for tumour initiation in their "stemness". Breast cancer stem cells (BCSCs) are a small portion of tumour-initiating cells within the multi-cellular tumour mass that possess self-renewing and tumour-initiating abilities (Raouf, Sun, Chatterjee, & Basak, 2012). The ability to form mammospheres is one of the defining features of BCSCs (Al-Hajj et al., 2003), and this ability is known to require anoikis resistance of the indicated cells (Kruyt & Schuringa, 2010). Moreover, anoikis resistance of BCSCs is considered necessary for prolonged survival of these cells during the process of detachment from the primary tumour site followed by invasion and metastasis (Kruyt & Schuringa, 2010; Shiozawa, Nie, Pienta, Morgan, & Taichman, 2013).

Perhaps not by coincidence, factors responsible for the “stemness” of BCSCs are well-known to block breast cancer cell anoikis (P. Paoli et al., 2013). For instance, sustained activation of growth factor receptors, such as EGFR (Berclaz et al., 2001),

ErbB2 (Jaime Lindsay et al., 2008) or Notch receptor, (Dontu et al., 2004) trigger anti-apoptotic pathways required for the ability of breast cancer cells to form mammospheres. To further support this data, Morel and group overexpressed components of the Mek-Erk signalling pathway (which mediates EGFR and ErbB2 signalling (Harari & Yarden, 2000)) in non-malignant breast epithelial cells which was observed to give rise to the cells with stem-cell like features (Morel et al., 2008). Furthermore, inhibition of the Mek/Erk signalling pathway induced anoikis sensitivity of the stem-cell like breast cancer cells (S. Y. Kim et al., 2016) indicating that anoikis resistance of BCSCs requires activation of the Mek/Erk signalling pathway.

1.8.3 Anoikis resistance as a critical pre-requisite for the ability of cancer cells to form tumours.

According to numerous studies, approaches that promote anoikis of breast cancer cells at the same time block their ability to form tumours *in vivo*. For example, ErbB2-mediated activation of hypoxia-inducible factor-1 (HIF-1) is required for anoikis resistance of breast cancer cells (Kelly A. Whelan et al., 2013). Furthermore, deletion of the HIF-1 gene was found to suppress ErbB2-driven tumour growth in mice (Kelly A. Whelan et al., 2013). Others demonstrated that the expression of E-cadherin, a protein known to mediate cell-cell adhesion, promotes anoikis of p53-deficient breast tumour cells (Derksen et al., 2006). It was further observed that homozygous deletion of the E-cadherin allele results in accelerated breast tumour growth in p53-deficient mice (Derksen et al., 2006).

Inhibition of anoikis resistance of tumour cells was observed to be paralleled by their reduced tumorigenicity in other types of cancer. For example, enforced downregulation of Bcl-X_L in malignant intestinal epithelial cells was shown to increase their anoikis sensitivity and was accompanied by reduced tumorigenicity in mice. Likewise, Bcl-X_L knockdown in ovarian carcinoma cells was also observed to promote their anoikis in tissue culture and suppress their ability to form tumours in mice.

1.8.4 Anoikis resistance as a critical pre-requisite for the ability of cancer cells to form metastases.

Cancer cell metastasis involves detachment of cancer cells from the primary site, invasion of the surrounding tissue, intravasation into the blood vessels, extravasation of the tumour cells into the surrounding tissues of distant organs and development of the secondary tumours (Langley & Fidler, 2011). Throughout these events cancer cells likely remain deprived of the proper contacts with the ECM yet at least some of them remain viable (P. Paoli et al., 2013).

Approaches targeting the ability of breast cancer cells to survive anchorage-independently were shown to suppress their ability to form metastases (Fiucci, Ravid, Reich, & Liscovitch, 2002; Sachdev, Zhang, Matise, Gaillard-Kelly, & Yee, 2009). For example, Derksen and group showed that the primary mammary tumour cells derived from E-cadherin and p53-deficient mice were able to survive without adhesion to the ECM and metastasized to the lungs when injected into the mammary fat pad of an immunodeficient mice (Derksen et al., 2006). In contrast, primary mammary tumour cells derived from p53-deficeint mice were more susceptible to anoikis *in vitro* compared to

tumour cells derived from E-cadherin and p53-deficient mice. The p53-deficient mammary tumour cells expressed E-cadherin and did not result into lung metastasis (Derksen et al., 2006). Similarly, Sachdev and group, have shown that pharmacological inhibition of type-1 insulin-like growth factor receptor (IGF1R) suppressed anoikis resistance of breast carcinoma cells *in vitro* (Sachdev et al., 2009). Moreover, combined injection of the indicated breast cancer cells along with antibody against IGF1R into the tail vein of nude mice suppressed their ability to form secondary metastases in the lungs of the mice compared to the mice without such treatment (Sachdev et al., 2009). It was found that inhibition of IGF1R activity in these breast cancer cells suppressed their survival in the blood circulation and thus inhibited their ability to form lung metastases in mice (Sachdev et al., 2009).

Based on these evidence, it can be suggested that anoikis resistance of breast epithelial cells is likely critical for their ability to undergo EMT, their stemness, the capacity for forming primary tumours and metastases.

1.9 The role of ErbB2 receptor tyrosine kinase in development of breast cancer.

Presently, classification of breast cancer is based on the status of estrogen receptor (ER), progesterone receptor (PR) and the Her2 (ErbB2) receptors (Masood, 2005; Vallejos et al., 2010). Breast cancer is classified into four molecular sub-types: Luminal A, Luminal B, Her2- positive and the basal breast cancer (Dai et al., 2015; Schnitt, 2010).

Luminal A tumours are ER positive (+), PR+ and Her2 negative (-) whereas luminal B are ER+, PR+ and HER2+/- (Sørlie et al., 2001; Yanagawa et al., 2012). Her2 is typically overproduced in breast cancers due to the ErbB2 gene amplification or increased ErbB2 protein stability whereas oncogenic mutations of ErbB2 gene are rarely found in breast cancers (Kourie, Rassy, Clatot, de Azambuja, & Lambertini, 2017). Her2-positive breast cancers also show a higher rate of cellular proliferation, lymph node metastasis and are poorly differentiated compared to other luminal cancers (Barnard, Boeke, & Tamimi, 2015; Kourie et al., 2017). Basal-like tumours are triple negative for ER, PR and Her2 receptors. These tumours are more aggressive than other molecular subtypes and display poor prognosis and very low disease-free survival rate (Dent et al., 2007).

1.10 ErbB2/Her2-positive breast cancer as a major breast cancer subtype.

ErbB2 (or Her2) is an oncoprotein that is overexpressed in 10 – 30% of breast cancer (Slamon et al., 1987; Slamon, Godolphin, Jones, Holt, Wong, Keith, Levin, Stuart, Udove, & Ullrich, 1989). In addition to breast cancer, ErbB2 also plays an important role in the progression of ovarian and lung carcinomas (Harari & Yarden, 2000; Slamon, Godolphin, Jones, Holt, Wong, Keith, Levin, Stuart, Udove, Ullrich, et al., 1989). ErbB2 gene amplification has been significantly considered as a prognostic marker that helps to predict disease-free and overall survival of the breast cancer patients (Slamon et al., 1987). In a retrospective study, it was shown that women with ErbB2-positive tumours were more prone to shorter relapse-free survival compared to hormone positive breast

tumours and thus was associated with early death of the patients (Carey et al., 2006; Zurawska et al., 2013).

Monoclonal anti-ErbB2 antibodies trastuzumab and pertuzumab in combination with chemotherapy are typically used for the treatment of ErbB2-positive breast cancer (Callahan & Hurvitz, 2011; Kourie et al., 2017). The fact that anti-ErbB2 antibodies can cure or slow down the progression of ErbB2-positive breast cancer indicates that this protein is a major driver of the disease. Not surprisingly, ErbB2 overexpression in the mammary cells is sufficient for triggering breast cancer in mice. Various transgenic mouse models expressing activated forms of ErbB2 under the transcriptional control of the mouse mammary tumour virus (MMTV) promoter have been generated (Muller, Sinn, Pattengale, Wallace, & Leder, 1988). These mice develop mammary tumours that metastasize to the lung (C. T. Guy et al., 1992).

1.11 ErbB Family of Receptor Tyrosine Kinases.

The members of the ErbB receptor tyrosine kinase family are 185 kDa membrane-spanning proteins that are comprised of four members: ErbB1 (or EGFR) (Ullrich et al., 1984), ErbB2 (Yamamoto et al., 1986), ErbB3 (Kraus, Issing, Miki, Popescu, & Aaronson, 1989) and ErbB4 (Plowman et al., 1993). All ErbB receptors have an extracellular ligand-binding domain (ECD), a transmembrane domain and an intracellular tyrosine kinase domain. When a ligand binds to its cognate ErbB receptor, it either induces homodimerization of the receptor or hetero-dimerization with the other ErbB members especially ErbB2. Dimerization of the ErbB receptors activates the intrinsic tyrosine kinase activity of the receptors (except for ErbB3) (Figure 8) which causes

autophosphorylation of specific tyrosine residues present in the cytoplasmic domain of these proteins (van der Geer, Hunter, & Lindberg, 1994). These phosphorylated tyrosine residues act as docking sites for the Src Homology 2 (SH2-) and phospho-tyrosine binding (PTB-) domain containing proteins such as the adaptor proteins SHC (Kavanaugh & Williams, 1994) and Grb2 (Lowenstein et al., 1992) as well as the p85 regulatory subunit of PI3K (Fedi, Pierce, di Fiore, & Kraus, 1994). Recruitment of these proteins results in the activation of signaling cascades such as those driven by Ras/MAPK (Egan & Weinberg, 1993) or PI3K/Akt (Carmona et al., 2016) required for cancer cell survival, proliferation and metastasis (N. E. Hynes & Stern, 1994).

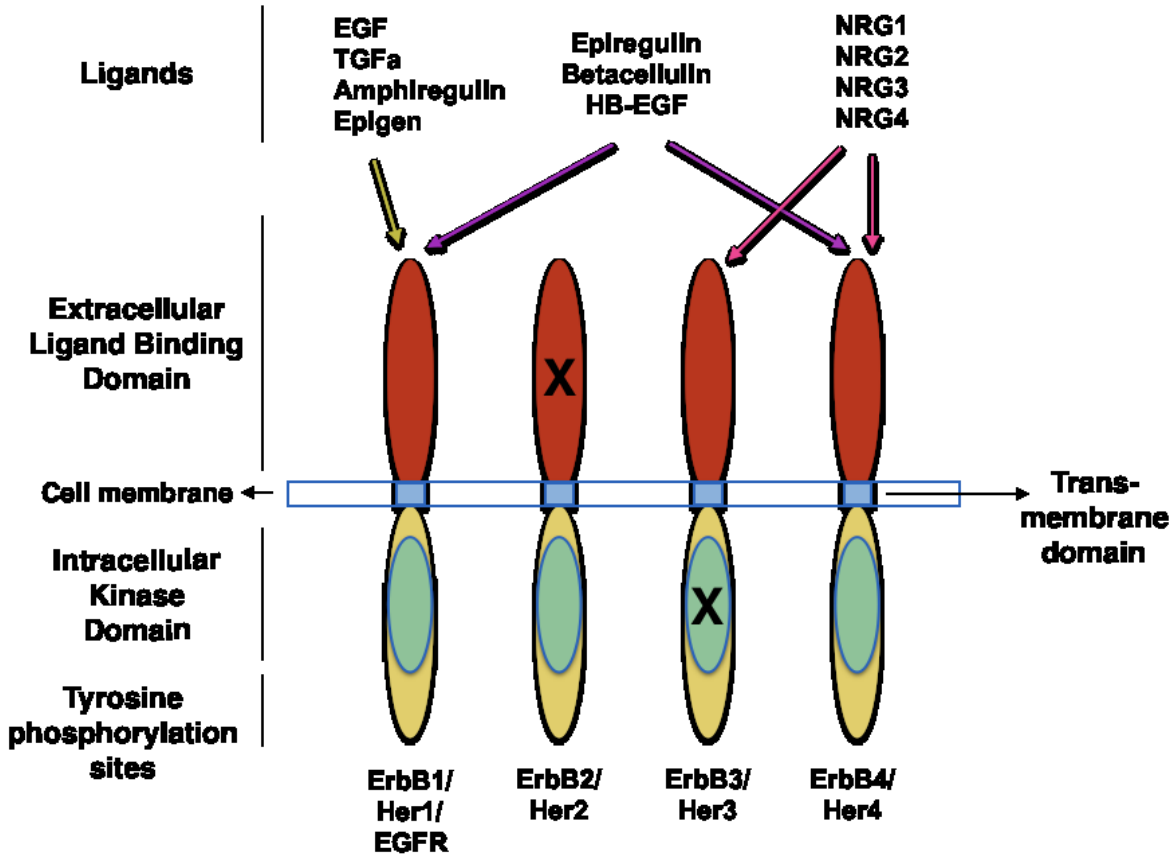

Figure 8. Schematic representation of the ErbB family of receptor tyrosine kinases. ErbB family of receptors is composed of four members: ErbB1/EGFR/Her1, ErbB2/Her2, ErbB3/Her3 and ErbB4/Her4. All the ErbB receptors contain the extracellular domain (shown in red), the transmembrane domain (shown in blue) and the intracellular domain (shown in yellow). The extracellular domain of all the members except for that of ErbB2 bind to the ligands shown in the figure. Some ligands can recognize more than one ErbB receptor. The intracellular domain of all the ErbB receptors except for that of ErbB3 contain a kinase domain (shown in green) is responsible for phosphorylating tyrosine residues present on the intracellular domain of their dimerizing partners (H. Zhang et al., 2007).

1.12 ErbB2-containing heterodimers trigger stronger survival signals.

ErbB2 is known as a powerful oncoprotein due to its ability to trigger stronger survival signals once it forms heterodimers with other ErbB members (Harari & Yarden, 2000). ErbB receptors are known to be inactivated by mechanisms that involve dissociation of ligand-receptor complexes, internalization of the receptor by the cells and subsequent lysosomal degradation of the receptor (Harari & Yarden, 2000). The mechanism by which EGFR undergoes lysosomal degradation is still unclear.

EGFR (ErbB1) activation followed by EGF-ligand binding was shown to recruit the adaptor protein (AP-2) which in turn recruits a clathrin molecule to the receptor (H. Chen, Slepnev, Fiore, & Camilli, 1999). AP2 also recruits cytosolic proteins Eps15 and epsin to itself for causing clathrin-mediated endocytosis (H. Chen et al., 1999). This causes invagination of the plasma membrane resulting in the formation of clathrin-coated vesicles (CCVs) (Tomas, Futter, & Eden, 2014). Activated EGFR containing CCVs cleave off from the membrane with the help of a GTPase enzyme called dynamin (Tomas et al., 2014). In the cytosol, the clathrin-coat is removed, and the vesicle forms an early endosome (Hirst & Robinson, 1998). The early endosome matures into the late endosome and fuses with the lysosomes for lysosomal degradation of EGFR (Hirst & Robinson, 1998). Many lines of evidence propose a role for receptor ubiquitination during endocytosis. An adaptor protein Grb2 can bind a specific phospho-tyrosine residue on the activated EGFR which then recruits an E3-ubiquitin ligase Cbl (Madshus & Stang, 2009). Cbl-mediated poly-ubiquitination of K63 lysine residue in the cytoplasmic domain EGFR was suggested to induce formation of CCVs followed by sorting of EGFR into the

lysosomes with the help of endosomal sorting complexes required for transporting (ESCRT) complexes (F. Huang et al., 2013; Madshus & Stang, 2009). Deubiquitination of active EGFR was proposed to promote recycling of the receptor and the deubiquitinating enzymes are overexpressed in human prostate and lung cancers (F. Huang et al., 2013). It should also be noted that EGFR ubiquitination sites were mapped to the kinase domain of the receptor (F. Huang, Goh, & Sorkin, 2007). Mutation of these sites were found to not affect EGFR internalization indicating that ubiquitination is not required for the indicated internalization (F. Huang, Goh, & Sorkin, 2007). Therefore, it is possible that Cbl controls EGFR internalization indirectly, for instance, by triggering ubiquitination of other proteins.

On the other hand, these events occur at a slower rate when the ErbB heterodimers contain ErbB2 compared to the dimers formed by other members of ErbB family that do not involve ErbB2 (Harari & Yarden, 2000). Such stability of ErbB2 was found to be induced by heat shock protein Hsp90 (Bertelsen & Stang, 2014). However, ErbB2 degradation can still be induced by inhibition of Hsp90 (Bertelsen & Stang, 2014). Hsp90 inhibitors, such as geldanamycin were found to recruit heat shock protein Hsp70 and ubiquitin ligase CHIP (C-terminus of Hsc70-interacting protein) to the cytosolic domain of ErbB2 (Castagnola et al., 2014). Hsp70 was proposed to induce conformational changes in the receptor thereby facilitating ubiquitination by CHIP (Castagnola et al., 2014). Ubiquitinated ErbB2 is then targeted for proteasomal as well as lysosomal degradation (Castagnola et al., 2014). Furthermore, inhibition of phosphatidylcholine-specific phospholipase C (PC-PLC) was also observed to induce lysosomal degradation of ErbB2 (Paris et al., 2010). PC-PLC associates with ErbB2 in

ErbB2 overexpressing breast cancer cells and inhibition of PC-PLC leads to endocytosis and lysosomal degradation of ErbB2 (Paris et al., 2010).

ErbB2-containing heterodimers transduce stronger signals than the dimers formed by the other members of the ErbB family in order to promote cellular proliferation and survival (Harari & Yarden, 2000). These signals involve activation of the MAPK and PI3K pathways, known to promote breast cancer progression (Bertelsen & Stang, 2014; Yarden & Sliwkowski, 2001).

1.13 Molecular Mechanisms of ErbB2 Signalling.

Several ligands can activate ErbB 1, 3 and 4 whereas ErbB2 has no known ligand. Therefore, ErbB2 is commonly known as the 'orphan receptor' (Nagy, Claus, Jovin, & Arndt-Jovin, 2010) and acts as a preferred dimerization partner for other ErbB receptors (R. R. Beerli et al., 1995; Roger R. Beerli & Hynes, 1996; Graus-Porta, Beerli, & Hynes, 1995). The abundance of ErbB2 in breast cancer cells allows the formation of ligand-induced heterodimers between ErbB2 and ErbB1 or between ErbB2 and ErBb3 receptors. (Kokai et al., 1989; Wallasch et al., 1995; X. Zhang et al., 2009). In addition, constitutively active ErbB2 homodimers may form due to single mutations in the transmembrane domain of the receptors but such mutations are uncommon in breast cancer (Olayioye et al., 1998; Weiner, Liu, Cohen, Williams, & Greene, 1989). ErbB1 and ErbB3/4 receptors are known to heterodimerize with ErbB2 in presence of their ligands EGF and neuregulin, respectively (Earp, Dawson, Li, & Yu, 1995). Although ErbB3 binds to a ligand, it lacks an active receptor tyrosine kinase activity in its

cytoplasmic domain and requires formation of heterodimers with other members of the ErbB family in order to trigger signalling events (Carraway & Cantley, 1994).

1.14 ErbB2-mediated signaling pathways that promote breast cancer.

Transactivation of ErbB2 by auto phosphorylation of its tyrosine residues creates binding sites for SH2 or PTB containing domains of signaling adaptor proteins such as Grb2 and Shc (Harari & Yarden, 2000; Senthil K. Muthuswamy, Gilman, & Brugge, 1999). Grb2 has one SH2 and two SH3 domains whereas Shc has one SH2 and one PTB domain (Wagner, Stacey, Liu, & Pawson, 2013). These adaptor proteins stimulate an assembly of downstream signalling complexes that promote cellular proliferation, survival and migration of cancer cells (Figure 9) (Uhlik et al., 2005; B. P. Zhou et al., 2000).

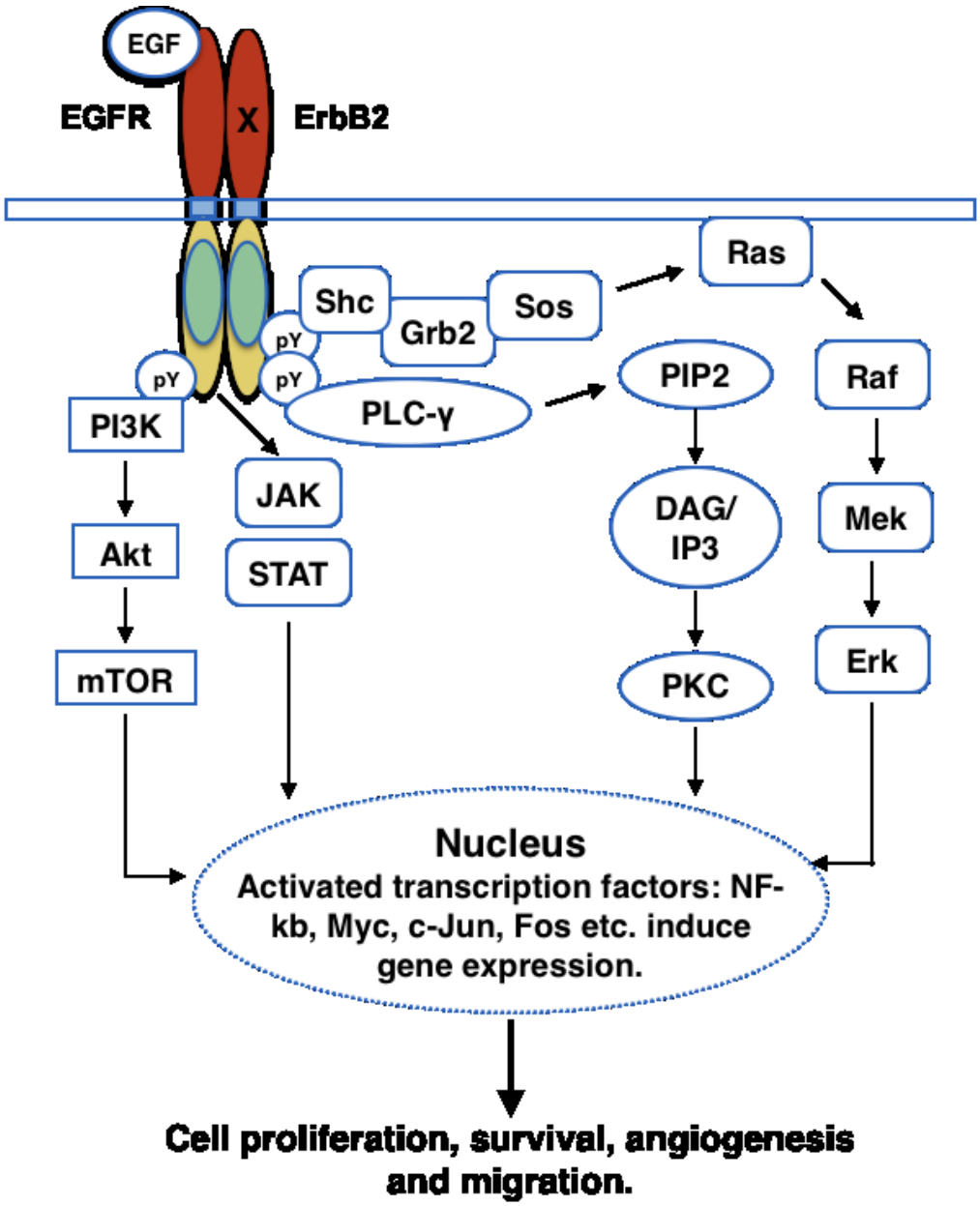

Figure 9. Schematic representation of the major signaling events induced by ErbB2. ErbB2 heterodimerizes with other ErbB receptors, such as EGFR, when the latter binds to its ligand (e.g. EGF). Heterodimerization activates the intracellular kinase domain which phosphorylates specific tyrosine residues in the cytoplasmic domain of the dimerizing partner and thus triggers activation of various signalling pathways such as those driven by PI3K/Akt, Jak-Stat, phospholipase C-ɣ and Ras. These mechanisms subsequently

promote expression of the genes involved in the regulation of cell growth, survival and migration (Modjtahedi, Cho, Michel, & Solca, 2014).

1.14.1 The Ras/MAPK/ERK Pathway

Ras proteins are guanosine-nucleotide-binding proteins also called as 'G proteins'. Harvey-Ras (Hras), Kirsten-Ras (Kras) and Neuroblastoma-Ras (Nras) are the three members of the Ras superfamily. The Ras proteins are inactive when they are bound to guanosine diphosphate (GDP) whereas binding to guanosine triphosphate (GTP) activates these proteins. Once Grb2, binds to the activated ErbB2, it recruits the guanine-nucleotide exchange factor known as Sos (son of seveless) through one of its SH3 domains (N. Li et al., 1993). Sos facilitates the exchange of GDP for GTP and thus activates Ras. Ras proteins have intrinsic GTPase activity which hydrolyzes the bound GTP molecule to GDP which inactivates Ras (Barbacid, 1987; Bos, 1989). Once activated, Ras further activates the serine-threonine kinase Raf which phosphorylates and activates a dual specificity kinase Mek. Active Mek phosphorylates and activates MAPK/ERK which phosphorylate and alter the activity of numerous proteins, including those that control cell proliferation and survival (Wagner et al., 2013). Oncogenic *ras* mutations are rarely observed in breast tumours (McLaughlin et al., 2013). However, Ras-dependent signalling pathways, such as those mediated by the Raf-Mek-Erk, PI3K/Akt or Ras-related Ral GTPases that are typically activated in ErbB2-positive breast cancer and contribute to its progression (Adeyinka et al., 2002; Eckert et al., 2004; Wright et al., 2015).

1.14.2 The Phosphatidylinositol 3-kinase/Akt/mTOR (PAM) pathway

Phosphatidylinositol-3 kinase (PI3K) belongs to a family of lipid kinases that are known to phosphorylate the 3'-OH group of inositol ring of the phosphatidylinositol lipids (Fruman, Meyers, & Cantley, 1998). PI3K is composed of a catalytic subunit (p110) and an adaptor/regulatory subunit (p85) (Vara et al., 2004). The p85 subunit of PI3K can either self-recruit themselves to the activated RTKs, such as ErbB2, (Reese & Slamon, 1997) through their SH2 domains or associate with Grb2 via Grb2 associated binder (Gab) proteins thereby inducing activation of its catalytic subunit (Hunter, 2000). The third way by which PI3K can get activated is via activation of p110 subunit of PI3K directly by Ras (Castellano & Downward, 2011; Rodriguez-Viciana et al., 1994). On activation, PI3K converts phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-triphosphate (PIP3) at the plasma membrane. PIP3 recruits protein kinase B/Akt kinase to the plasma membrane where 3-phosphoinositide-dependent protein kinases 1 and 2 (PDK1 and 2) phosphorylate and activates the PKB/AKT kinase (Newton, 2009). AKT phosphorylation triggers phosphorylation of numerous targets by Akt, such as a serine/threonine kinase mammalian target of rapamycin (mTOR) that regulates cell growth, proliferation, motility, survival and protein synthesis (Hay & Sonenberg, 2004; Yang, Polley, & Lipkowitz, 2016). PI3K/Akt pathway can be attenuated by the activity of a phosphatase PTEN which dephosphorylates PIP3 to PIP2 (Vara et al., 2004).

20 – 25% of breast cancers overexpressing ErbB2-containing heterodimers can activate PI3K pathway (Holbro et al., 2003). Moreover, some ErbB2-positive breast cancers display activating mutations of the PI3K gene, PIK3CA or loss-of-function

mutations of the PTEN gene (Hennessy et al., 2009; J. J. X. Lee, Loh, & Yap, 2015; Stemke-Hale et al., 2008).

1.14.3 The Phospholipase C-gamma (PLC-γ) pathway

The phosphoinositide specific phospholipase C-γ (PLC-γ) binds to the activated ErbB heterodimers containing ErbB2 receptor through its SH2 domain (Senthil K. Muthuswamy et al., 1999; Peles, Levy, Or, Ullrich, & Yarden, 1991). Its catalytic activity is triggered by phosphorylation of its regulatory tyrosine residues by the activated RTKs (H. Kim et al., 1991; Margolis et al., 1990). Activated PLC-γ cleaves PIP2 to generate secondary messengers inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 is responsible for regulating the intracellular calcium ions levels and DAG activates protein kinase C (PKC) by binding to its regulatory subunit (Noh, Shin, & Rhee, 1995). Activation of PKC leads to activation of various signalling pathways that induce cell proliferation and survival. For instance, active PKC can activate survival mechanisms such as Mek-Erk and Akt signaling pathways (Basu & Sivaprasad, 2007). In addition, PKC can activate Rho-GTPases which can trigger numerous responses, including enhanced cell motility. PKC was also observed to inhibit pro-apoptotic protein Bad-induced apoptosis by activating p90RSK (p90 Ribosomal S6 Kinase), a downstream target which on activation causes phosphorylation of Bad. Phosphorylated Bad is sequestered by 14-3-3 protein in the cytoplasm which blocks binding of Bad to Bcl-X_L and thus promotes cell survival and motility (Bertolotto, Maulon, Filippa, Gottfried, & Auberger, 2000; Zha, Harada, Yang, Jockel, & Korsmeyer, 1996).

1.14.4 The Src kinase pathway

The Src family of protein kinases (SFKs) are cytosolic tyrosine kinases that play a major role in transducing signals from the cell surface receptors to the cell nucleus and promote tumourigenesis and metastasis (Martin, 2001). There are nine members in the Src family of kinases: c-Src, Yes, Fyn, Yrk, Blk, Fgr, Hck, Lck, Lyn and Frk. In metastatic breast cancer, Active ErbB2 receptor was shown to activate cellular Src (c-Src) kinases by directly binding to the SH2 domain of Src kinases through its phosphorylated tyrosine residues (Luttrell et al., 1994; S. K. Muthuswamy & Muller, 1995; Sheffield, 1998; M. Tan et al., 2005). Src kinase activation leads to the activation of the PI3K pathway by either phosphorylating tyrosine residues in the cytoplasmic domain of the cell surface receptor ErbB1 (EGFR) (Yarden & Sliwkowski, 2001)which acts as a docking site for the p85 regulatory subunit of PI3K (Biscardi, Ishizawar, Silva, & Parsons, 2000). Src can also activate PI3K directly by binding to the p85 regulatory subunit of PI3K (Pleiman, Hertz, & Cambier, 1994). This further potentiates signaling from EGFR receptor (Stover, Becker, Liebetanz, & Lydon, 1995). The Src kinases have also been implicated in transactivation of ErbB2 by integrins. ErbB2 phosphorylates β4 integrins through c-Src activation which further phosphorylates ErbB2 resulting into increased activity of the ErbB2 receptor (Guo et al., 2006). Other downstream effectors of the Src kinases are Grb2, focal adhesion kinases, the Janus kinases and signal transducer and activator of transcription (JAK-STAT) etc. that are required for mammary carcinogenesis (Sen & Johnson, 2011).

1.14.5 The JAK-STAT pathway

The STAT proteins are a family of transcription factors that can be activated by ErbB2 via activation of Src kinases and/or JAK protein kinases (Z. Ren & T. S. Schaefer, 2002). The JAK kinases are phosphorylated and activated by growth factor receptors, such as EGFR or ErbB2 (Zhiyong Ren & Timothy S. Schaefer, 2002) or by the non-receptor tyrosine kinases, such as Src (Bowman, Garcia, Turkson, & Jove, 2000; Rawlings, Rosler, & Harrison, 2004). Active growth factor receptors like EGFR are also known to directly activate the STATs (Bowman et al., 2000). Once phosphorylated, the STATs dimerize and enter the cell nucleus as active transcription factors where they stimulate transcription of numerous genes, including those that promote cancer (Bromberg & Darnell, 2000). It was observed that ErbB2-induced STAT3 activation enhanced the invasiveness and tumourigenic capacity of BCSCs (Duru et al., 2012).

1.14.6 The Notch-signaling pathway

Notch are large transmembrane proteins that act as receptors for the Jagged/Delta family of ligands. Notch family of receptors consists of four members, Notch 1 – 4 (Brennan & Brown, 2003; Dontu et al., 2004). Notch proteins are composed of the extracellular domain, the transmembrane domain and the intracellular domain. Ligand binding of Notch results in proteolytic cleavage of the intracellular domain by the ɣ-secretase enzyme which releases Notch intracellular domain (NICD). NICD translocates to the nucleus where it binds the CSL (CBF1, Suppressor of Hairless, Lag-1) transcription factor (Pursglove & Mackay, 2005). This NICD-CSL complex activates transcription of genes involved signaling pathways that promote cell proliferation and

survival in breast epithelial cells (Al-Hussaini, Subramanyam, Reedijk, & Sridhar, 2011; Pursglove & Mackay, 2005).

Notch signaling is often reported to be activated in breast cancers (Stylianou, Clarke, & Brennan, 2006) whereas NUMB (protein numb homologue) a negative regulator of Notch signaling acts as a suppressor of breast cancer progression (Pece et al., 2004; J. Zhang et al., 2016). Notch proteins were observed to be overproduced by ErbB2 overexpressing breast cancer cells (Jaime Lindsay et al., 2008). It was observed that ErbB2-mediated activation of Notch 1 occurred via activation of cyclin D1 (a cell cycle regulatory protein (Kato, Matsushime, Hiebert, Ewen, & Sherr, 1993)) which in turn causes suppression of NUMB in breast epithelial cells (Jaime Lindsay et al., 2008). ErbB2-induced activation of Notch pathway was further found to promote anoikis resistance of ErbB2-overproducing breast epithelial cells (Jaime Lindsay et al., 2008; Pradeep et al., 2011). In addition, ErbB2-mediated activation of Notch signaling pathway was found to result in the luminal filling of mammary acini *in vitro* as well as promote mammary tumour formation in mice expressing the oncogenic mutant form of ErbB2 (Pradeep et al., 2011).

1.15 ErbB2 as an activator of the network of the anti-anoikis signals.

ErbB2 has been reported to block anoikis resistance of breast cancer cells (Nanni et al., 2000) but the mechanisms of this effect of ErbB2 are partially understood.

1.15.1 ErbB2-mediated upregulation of EGFR as an anti-anoikis mechanism.

Further to detachment of breast epithelial cells from the ECM, EGFR was shown to form a complex with a ring-finger domain E3 ubiquitin ligase, Cbl. Cbl was observed

to facilitate lysosomal degradation of EGFR (Levkowitz et al., 1999). ErbB2 was demonstrated to block this degradation by upregulating a protein Sprouty-2 which sequesters Cbl and thus blocks its binding to the activated EGFR. This mechanism of EGFR stabilization was shown to block anoikis of ErbB2-overproducing breast cancer cells (Grassian, Schafer, et al., 2011).

1.15.2 ErbB2-mediated downregulation of Bim as an anti-anoikis mechanism.

Reginato et al. showed that detachment of non-malignant breast epithelial cells causes loss of EGFR which increases expression of pro-apoptotic factor Bim (M. J. Reginato et al., 2003). Thus, upregulation of Bim promotes anoikis of these cells (M. J. Reginato et al., 2003). It was further observed that ErbB2 overexpression activated Mek-Erk signalling pathway which induced downregulation of Bim and promoted anoikis resistance of detached breast epithelial cells (M. J. Reginato et al., 2003). Moreover, ErbB2-mediated downregulation of Bim was shown to disrupt the lumen formation within the acini-like structures formed by these cells in culture (see Section 1.3.1 page 5) (Mauricio J. Reginato et al., 2005). In addition, ErbB2-mediated downregulation of Bim expression through the Mek-Erk signalling pathway was found to be dependent upon the upregulation of integrin $\alpha 5$ and subsequent activation of the Src family kinases in these cells (Haenssen et al., 2010).

1.15.3 ErbB2-mediated downregulation of Bmf as an anti-anoikis mechanism.

Anoikis of breast cancer cells was also found to be blocked by ErbB2-dependent downregulation of a pro-apoptotic Bcl-2 family member Bmf (Schmelzle et al., 2007). Activated Mek or Akt were demonstrated to be able to mimic the effect of ErbB2 on

Bmf (Schmelzle et al., 2007). In addition to this mechanism, hypoxia-mediated activation of HIF-1 was observed to suppress Bmf expression in breast epithelial cells which eventually inhibited luminal clearance within mammary acini (K. A. Whelan et al., 2010). Since ErbB2 was found to stabilize HIF-1 expression, it was suggested that ErbB2-mediated downregulation of Bmf requires activation of HIF-1 to promote anoikis resistance of breast cancer cells (Kelly A. Whelan et al., 2013).

In summary, ErbB2 blocks anoikis of breast cancer cells by activating a complex network of signals. How various elements of this network interact with each other functionally and whether all elements of the network have been identified is not known. Our goal is to further understand the mechanisms by which ErbB2 blocks anoikis of breast cancer cells.

1.16 Concluding Remarks

Every year 400,000 patients diagnosed with breast cancer die worldwide (Brollo et al., 2013; Patani & Mokbel, 2010). ErbB2 receptor is overexpressed in 10 - 30% of breast carcinoma and is associated with poor prognosis (Ross et al., 2009; Slamon et al., 1987; Slamon, Godolphin, Jones, Holt, Wong, Keith, Levin, Stuart, Udove, & Ullrich, 1989; Vogel, 2010). Treatments involving monoclonal antibodies against ErbB2 have provided significant benefit to patients with ErbB2-positive breast cancer (Singh, Jhaveri, & Esteva, 2014). However, a significant fraction of these patients succumbs to the disease. Understanding the mechanisms by which ErbB2 blocks breast cancer cells anoikis resistance, a critical property of breast cancer cells, could give rise to the novel

treatments of ErbB2-positive cancer and prognostic or predictive markers of the efficacy of ErbB2-trageted therapies.

1.17 Hypothesis and Objectives

The main hypothesis of this study is that ErbB2 blocks anoikis of breast cancer cells by altering the expression and/or activity of proteins that control apoptosis. In an effort to test this hypothesis we found that ErbB2 downregulates the pro-apoptotic proteins Perp and Irf6 in breast cancer cells and stabilizes its own expression in breast cancer cells detached from the ECM by activating the MAPK signalling pathway. These findings have been described in detail in following chapters. To address our questions, we have focused on the following objectives:

Specific Objective I

To test whether ErbB2-dependent downregulation of Perp blocks anoikis of breast cancer cells.

Specific Objective II

To test whether ErbB2-dependent downregulation of Irf6 blocks anoikis of breast cancer cells.

Specific Objective III

To test whether ErbB2-induced stabilization of its own expression in breast cancer cells detached from the ECM is required for their anoikis resistance.

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Compounds (see Appendix B for catalogue numbers and working concentrations)

The following compounds were used in this study. Lapatinib Dictosylate and SCH772984 (Selleckchem, Houston, TX, USA), PD98059 (Sigma, St Louis, MO) and LY294002 (Sigma, St Louis, MO), Selumetinib (Santa Cruz Biotechnology, Dallas, TX or ApexBio, Houston, TX), Bafilomycin A1 (Sigma, St Louis, MO), cycloheximide (Sigma, St Louis, MO), MG132 (Calbiochem, Etobicoke, ON), trastuzumab (Roche, Mississauga, ON), polybrene (Sigma, St Louis, MO).

2.1.2 Cell culture medium and reagents (see Appendix B for catalogue numbers)

Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand island, NY), Dulbecco's Modified Eagle Medium F12 (DMEM/F12) (Gibco, Grand island, NY), Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Grand island, NY), Hybri-Care medium (ATCC, Manassas, VA), McCoy's 5a medium (Gibco, Grand island, NY), Mammary Epithelium Basal Medium (MEBM) and Mammary Epithelium Cell Growth Medium (MEGM) SingleQuots (Lonza, Walkersville, MD), horse serum (Gibco, Grand island, NY), fetal bovine serum (Seradigm, USA), hEGF (Invitrogen, Carlsbad, CA), cholera toxin (Biolynx Inc, Brockville, ON) hydrocortisone (Sigma, St Louis, MO), penicillin-streptomycin (Gibco, Grand island, NY) L-glutamine (Gibco, Grand island, NY), insulin (Novolin, GE Toronto, Mississauga, ON), Hygromycin B (Invitrogen, Carlsbad, CA), Zeocin (Invitrogen, Carlsbad, CA), Puromycin (Gibco, Grand Island,

NY), Lipofectamine 2000 (Invitrogen, Carlsbad, CA), Opti-MEM (Gibco, Grand island, NY), Bacto Agar (BD, Sparks, MD), N, N-bis (2 hydroxyethyl)-2- aminoethanesulfonic acid (BES) (Sigma, St. Louis, MO), sea plaque agarose (Lonza, Walkersville, MD), 25X protease inhibitor cocktail (Roche, Mississauga, ON), Taq DNA polymerase (Invitrogen, Carlsbad, CA), T4 DNA ligase Buffer (New England Biolabs, Ipswitch, MA), Apoptosis Detection kit (Millipore, Billerica, MA), 30% Acrylamide/Bis Solution 37.5:1 (Bio-Rad, Hercules, CA), sodium vanadate (Sigma, St Louis, MO), phenylmethylsulfonyl fluoride (PMSF) (Sigma, St Louis, MO), Protease inhibitor cocktail (Roche Diagnostics, Indianapolis), Ammonium persulfate (Sigma, St Louis, MO), Tetramethylethylenediamine (TEMED) (Bio-Rad, Hercules, CA), Tween-20 (Sigma, St Louis, MO), Immobilon-P Polyvinylidene difluoride membrane (PVDF) (Millipore, Darmstadt, Germany), bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL), Bovine serum albumin (Sigma, St Louis, MO), Enhanced chemiluminescence reagent (GE lifesciences, Pittsburgh, PA or Millipore, Billerica, MA), X-ray films (Mandel scientific, Guelph, ON).

2.2 Methods

2.2.1 Cell culture

MCF10A (ER positive, PR positive, HER2 negative and TP53 positive) cells and their derivatives MCF10A-vector (pBabe), MCF-ErbB2 (pBabe-ErbB2) and MCF-MekDD (S222D/S226D) (pBabe Mek-2-DD) were provided by Dr. M Reginato (Drexel University, Philadelphia, PA, USA) (Haenssen et al., 2010; Reginato et al., 2003). MCF10A cells were authenticated by American Type Culture Collection, Manassas, VA,

USA by seventeen short tandem repeat analysis. The cells listed above were cultured in the medium containing DMEM/F12 supplemented with 5% horse serum, 20 ng/ml hEGF, 10 μg/ml insulin, 0.1 μg/ml cholera toxin, 0.5 mg/ml hydrocortisone, 50 U/ ml penicillin, 50 μg/ml streptomycin, 0.146 mg/ml L-glutamine. BT-474 (ER positive, PR positive, HER2 positive and TP53 mutant positive) (ATCC, Manassas, VA) were cultured in Hybri-Care medium, 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.29 mg/ml L-glutamine. AU-565 (ER negative, PR negative, HER2 positive and TP53 mutant positive) (ATCC, Manassas, VA), SKBR3 (ER negative, PR negative, Her2 positive and TP53mutant positive) were cultured in McCoy's5a medium, 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin (ATCC, Manassas, VA) and HCC1419 (ER positive, PR negative, HER2 positive and TP53 mutant positive) (ATCC, Manassas, VA) were cultured in RPMI1640 medium, 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.29 mg/ml L-glutamine. The primary human mammary epithelial cells HMEC (Lonza, Walkersville, MD) were cultured in mammary epithelial growth medium MEBM supplemented with bovine pituitary extract (BPE), hEGF, insulin, hydrocortisone, gentamicin (30 mg/ml) and amphotericin (15 mg/ml) also known as MEGM. 293T cells (provided by Dr. A. Stadnyk, Dalhousie University, Halifax, NS, Canada) were cultured in DMEM, 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.29 mg/ ml L-glutamine. The cells were detached from ECM, by culturing them over a layer of 1% sea plaque agarose polymerized in respective culture medium devoid of any other additional ingredients.

2.2.2 Western blot analysis (see Appendix B for catalogue numbers and final dilutions of antibodies).

Cells were harvested and lysed as described previously (Rosen, Shi, Calabretta, & Filmus, 2002). For western blot analysis of protein expression, cells were lysed, and protein concentration was determined by using the BCA protein assay kit. The cell lysates were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P Polyvinylidene difluoride membrane. The membranes were incubated in 5% skimmed milk or BSA dissolved in 0.1% TBST (Tris buffer saline with Tween-20). The following are the antibodies that were used in this study: Anti-Perp and anti-Sprouty2 antibody is from Abcam (Cambridge, MA), Anti-Irf6, anti-ErbB2/Her2, anti-Akt1, anti-phospho-Akt (Ser 473), anti-MAPK/Erk, anti-phospho-MAPK/Erk, anti-Mek1, anti-RSK, anti-phospho-RSK and anti-GAPDH antibodies were from Cell Signalling Technology Danvers, MA, USA), anti-ΔNp63 was from Biolegend (San Diego, CA), anti-EGFR, anti-CDK4 were from Santa Cruz Biotechnology (Santa Cruz, CA), anti-β-actin was from Santa Cruz Biotechnology (Santa Cruz, CA) and Sigma (St. Louis, MO), anti-tGFP (OriGene Technologies, Rockville, MD), anti-Rabbit IgG was obtained from and Anti-Mouse IgG were purchased from Bio-Rad (Hercules, CA). Antibody catalogue numbers are indicated in Appendix B. The membranes were treated with chemiluminescence reagent (GE lifesciences, Pittsburgh, PA) and proteins were visualized by use of the X ray film (Mandel scientific, Guelph, ON).

2.2.3 RNA interference

RNA interference was performed as described (Liu et al., 2006). The sequences of the sense strands of the RNAs used in this study were as follows: control RNA (siCONTROL non-targeting siRNA #1 (Dharmacon, Lafayette, CO, USA), UGUUGUUUGAGGGGAACGGTT; ErbB2 siRNA 17, UGGAAGAGAUCAC AGGUUA; ErbB2 siRNA 18, GAGACCCGCUGAACAAUAC; Perp siRNA 5, GUCAGAGCCUCAUGGAGUA; Perp siRNA 7, GAAAUGCUCCCAAGAGGGC; EGFR siRNA 10, CAAAGUGUGUAACGGAAUA; EGFR siRNA 11, CCAUAAAUGCUACGAAUAU; Sprouty-2 siRNA 7, CCACUGAGCAAGGAAGAUU; Sprouty-2 siRNA 10, CAACAUAGCAUCAUUAAUC; Irf6 siRNA 5, GGAAACUCA-UCUUGGUUCA; Irf6 siRNA 7, CGUUUGAGAUCUACUUAUG; TP63 siRNA 14, CGACAGUCUUGUACAAUUU; TP63 siRNA 15, GAUGAACUGUUAUACUUAC. All the RNAs were purchased from Dharmacon.

2.2.4 Expression vectors

The expression vector encoding the activated Mek mutant was provided by M. Reiginato (Haenssen et al., 2010; Khan et al., 2016). C-terminal GFP-tagged human Perp encoded in pCMV6-AC-GFP expression vector (GFP-Perp) was purchased from Origene (Rockville, MD, USA). pEGFP-C1 plasmid encoding wild-type GFP was from Clontech (Mountain View, CA, USA). To generate MCF-ErbB2-Perp cells the mouse Perp cDNA was PCR amplified and cloned into pLEX-MCS lentiviral vector packaging vectors were provided by members of the lab of Dr. Laura Attardi (Stanford University, CA, USA).

pHIT and pVSVG retroviral vectors were provided by Dr. P. Lee (Dalhousie University, Halifax, NS, Canada), pBabe- hygro expression vector was from Addgene (Cambridge, MA, USA) The expression vector pcDNA-HA encoding a full-length human Irf6 cDNA was provided by Dr. Antonio Costanzo (University of Rome Tor Vergata, Rome, Italy) (Botti et al., 2011; Moretti et al., 2010). The pcDNA expression vector encoding the full length human ΔNp63alpha-FLAG was a gift from David Sidransky (Chatterjee et al., 2008) (purchased from Addgene, Cambridge, MA, USA). pBabehygro-HA-Irf6 and pBAbehygro-ΔNp63alpha-FLAG expression vectors were generated by Dr. BH Yoo (Dalhousie University, Halifax, Canada).

To insert the HA-Irf6 cDNA fragment into the pBabe-hygro expression vector the HA-Irf6 cDNA carrying SalI restriction sites at both ends was generated by PCR using pcDNA-HAIrf6 expression vector described above as a template and the oligonucleotides TTAGTCGACATGGCTTACCCA as a forward primer, and TTAGTCGACTTACTGGGGAGG as a reverse primer. The resulting PCR product was purified in 0.8% agarose gel using QIA quick Gel Extraction Kit (Qiagen, Toronto, ON). The amplified HA-Irf6 cDNA fragment was ligated into the pCR2.1-TOPO vector using TOPO TA Cloning kit (Invitrogen, Carlsbad, CA), and the insert was sequenced using the M13 forward primer GTAAAACGACGGCCAG and the M13 reverse primer CAGGAAACAGCTATGAC at Clinical Genomic Centre at the Mountain Sinai Hospital (Toronto, ON, Canada). The vector was digested with SalI restriction enzyme (New England Biolabs, Whitby, ON), the resulting Irf6 cDNA was purified and extracted from 0.8% agarose gel. The SalI-digested pBabe-hygro expression vector was dephosphorylated by alkaline phosphatase (New England Biolabs, Whitby, ON). The

indicated HA-Irf6 cDNA was ligated to the SalI-digested pBabe-hygro vector by use of T4 DNA ligase (New England Biolabs, Whitby, ON). The *E. coli* competent cells were transformed with the ligation mixture, the DNA was extracted from the resulting colonies and the presence of the HA-Irf6 cDNA was confirmed by digestion of the indicated vector with the EcoRI restriction enzyme.

To generate ΔNp63α-encoding retrovirus ΔNp63α-FLAG cDNA with BamHI and SalI restriction sites at 5' and 3' ends respectively was generated by PCR using the forward primer TTAGGATCCATGTTGTACCTG, the reverse primer TATGTCGACCTACTACTTGTCATCGTC and the pcDNA ΔNp63α-FLAG as a template. The PCR product was purified in a 0.8% agarose gel as above. The pBabe-hygro vector and the indicated ΔNp63α-FLAG DNA fragment were digested with BamHI (New England Biolabs, Whitby, ON) and SalI (New England Biolabs, Whitby, ON) restriction enzymes, the digested fragments were purified in a 0.8% agarose gel as above and ligated to each other by T4 DNA ligase. The *E. coli* competent cells were further transformed with the ligation mixture, the DNA was extracted from the resulting colonies and the presence of the ΔNp63α-FLAG in the vector was confirmed by sequencing using the sequencing primers CCGTCTCTCCCCCTTGAAC, GCAGAACAGCGTCACGGC, GCCCAGTATGTAGAAGATCCCA, and GGGCCGT GAGACTTATGAAA at the Clinical Genomics Centre at the Mountain Sinai Hospital (Toronto, ON, Canada). pHIT and pVSVG retroviral vectors were provided by Dr. P. Lee (Dalhousie University). pBabe-hygro retroviral expression vector was from Addgene.

2.2.5 qPCR (quantitative polymerase chain reaction)

This procedure was performed as described previously (Yoo et al., 2011). The mRNA extraction kit was from Qiagen (Maryland, USA) and the Reverse Transciption kit was from Clontech (Mountain View, CA). Primers used to amplify the Perp mRNA were as follows: GCATGAAGGGTGAAGGTCTG and GATGCTTGTCTT CCTGAGAGTG. Primers used to amplify the ErbB2 mRNA were as follows: ACCTGCTGAACTGGTGTATG and TGACATGGTTGGGACTCTTG. The primer sequences used to amplify Irf6 mRNA were as follows: CAAAACTGAACCCCTGGAGATGGA and CCACGGTACTGAAAC-TTGATGTCC. Primers used to amplify the 18 S rRNA used as control were as follows: ATAGTCAAGTTCGACCGTCTTC and GTTGATTAAGTCCCTGCCCTT.

2.2.6 Transient transfections assays

5×10^5 MCF-ErbB2 cells grown in 60-mm dishes were cultured for 6 h in the presence of 2 μg of either pEGFP-C1 or Perp-GFP expression vectors and 2 μl of Lipofectamine 2000 in 2 ml of Opti-MEM after which the medium was replaced with 4 ml of fresh medium normally used for culturing these cells. The cells were cultured further for 24 h, harvested and used for subsequent assays.

In case of the double transfection assays, 5×10^5 MCF-ErbB2 cells grown in 60-mm dishes were cultured for 6 h in the presence of 4 μg of either pcDNA-HA or pcDNA-HA-Irf6 along with pEGFP-C1 plasmid DNA in 5:1 ratio and 4 μl of Lipofectamine 2000 in 2.0 ml of Opti-MEM. After 6 h, the medium was replaced with 4 ml of fresh medium

normally used for culturing these cells. The cells were cultured in suspension for further 72 h, harvested and used for subsequent assays.

2.2.7 Generation of Perp-overproducing cells

5 μg of either pLex or Perp-encoding pLex vectors, 2.5 μg of pMD2.G expression vector and 2.5 μg of pSPAX2 expression vector encoding lentiviral proteins were mixed with 45 μl of 2.5 M $CaCl_2$. The mixture was added to 450 μl of a buffer containing 50 mM N, N-bis (2 hydroxyethyl)-2- aminoethanesulfonic acid, 280 mM NaCl and 0.1 M sodium phosphate (pH 6.95). The mixture was incubated for 30 min at room temperature and added to 2×10^6 Hek293T cells together with 2.5 μl of 100 mM chloroquine. Cells were cultured for 6 h, after which the medium was replaced with the regular culture medium. The medium was collected from the cells 48 h later, filtered through a 0.45-micron filter unit. Five milliliters of the resulting mixture were added to 3 × 105 MCF-ErbB2 cells in the presence of 8 μg/ml polybrene, and the cells were cultured for 48 h. Culture medium was changed to the regular culture medium for 48 h. Zeocin (650 μg/ml) was added to the cells for selection. Zeocin-resistant cells were expanded and used for subsequent studies.

2.2.8 Transduction of cells with retroviruses encoding HA-Irf6 or DeltaNp63alpha-FLAG.

2×10^6 293 T cells were transfected with 5 μg of either control pBabehygro expression vector or pBabehygro-HA-Irf6 expression vector or pBabe-deltaNp63alpha-FLAG expression vector and 2.5 μg of pHIT and 2.5 μg of pVSVG expression vectors encoding retroviral proteins in the presence of 20μl of Lipofectamine 2000 (Invitrogen,

Thermo Scientific) in 6 ml of Opti-MEM medium. The medium was changed after 4 h to DMEM containing 10% FBS. The medium was collected 48 h later and filtered through a 0.45-micron filter unit. 1 ml of the viral supernatant containing viruses encoding either empty vector or HA-Irf6 was used to infect 2.5×10^5 MCF10A cells grown on a 60-mm dish in the presence of 8 μg/ml polybrene. 2 ml of the viral supernatant containing viruses encoding either empty vector or ΔNp63alpha-FLAG was used to infect 2.5×10^5 MCF-ErbB2 cells respectively grown on a 60-mm dish in the presence of 8 μg/ml polybrene After 48 h of incubation, the medium was changed to fresh medium. The infected MCF10A and MCF-ErbB2 cells were cultured in the presence of 200 and 450 μg/ml hygromycin respectively for 48 h followed by cell harvest and used for subsequent assays.

2.2.9 Generation of trastuzumab-resistant breast cancer cell line.

1×10^6 BT-474 cells were cultured in suspension for two weeks in the presence of 5 μg/ml trastuzumab. The surviving cells were maintained in the monolayer culture in the presence of 5 μg/ml trastuzumab for four months.

2.2.10 Cell survival analysis

a) Soft agar growth assay

10^3 cells were suspended in 2 ml of their growth medium in the presence of 10% fetal bovine serum containing 0.3% melted Bacto agar. The resulting suspension was added to a 60-mm plate covered with a 2-ml layer of solidified 0.5% Bacto agar dissolved in the same medium. Cell colonies were allowed to form for 10 to 20 days and counted (Rosen, Shi, Calabretta, & Filmus, 2002a).

b) Clonogencity assay

100 cells/ml were placed in suspension culture for the indicated times and plated on 60-mm tissue culture dishes. Cell colonies were allowed to form for 1-2 weeks and counted after Crystal Violet staining (Rosen et al., 2002b). To test the effects of Irf6 on apoptosis in adherent cells, the MCF10A cells expressing either pBabehygro or pBabehygro-HA-Irf6 were trypsinized and counted after the selection. 250 cells were re-plated on a 60-mm dish. The cells were allowed to grow, and form colonies for 7-10 days followed by Crystal Violet staining.

c) Annexin-V binding assay

Apoptosis Detection kit was used for the assay according to manufacturer's instructions. FACS Calibur system (BD Biosciences) was used for the analysis (Yoo, Berezkin, Wang, Zagryazhskaya, & Rosen, 2012).

d) Detection of apoptotic nuclei by fluorescence microscopy.

Cells cultured in suspension were harvested, trypsinized and washed in phosphate buffered saline followed by centrifugation at 1500 rpm for 5 minutes at room temperature. The cell pellet was re-suspended and fixed in 50 µl of 4% paraformaldehyde solution for 30 min of incubation at room temperature. The cells were washed and re-suspended in 30-50 µl of 20 µg/ml Hoechst stain (Molecular Probes, Eugene, OR) dissolved in PBS. Tubes with cell samples were coded to ensure that the samples are assayed blindly, and the cells were analysed by the Zeiss fluorescence microscope (Carl Zeiss, Toronto, ON) for the presence of condensed or fragmented nuclei (Rosen et al., 2002a).

e) Cell counting assay

Cells were cultured in suspension, harvested at various time points and counted by using the hemocytometer (Yoo et al., 2010).

2.2.11 Statistical analysis

The exact sample size (n) for each experiment, a description of the sample collection, P-values, definition of center values and error bars is shown in each respective figure legend. Unless specified otherwise, every experiment was performed twice with similar results. Statistical analysis of all other data was performed by the unpaired Student's t-test or chi-square test for goodness-of-fit.

2.2.12 Image processing.

Western blot images were generated by Photoshop. When lanes were removed from western blot images, and separate parts of an image were joined together, a short vertical black line was used to indicate where the image was cut.

CHAPTER 3. RESULTS

3.1 ErbB2-dependent downregulation of a pro-apoptotic protein Perp is required for oncogenic transformation of breast epithelial cells.

The text of this chapter is presented verbatim as it was published by us (I. A. Khan et al., 2016) with the exception of those cases when the text was modified according to the Dalhousie University Thesis guidelines. See Appendix A for author contributions and Appendix C for the permission from Oncogene.

3.1.1 ErbB2 Downregulates Perp in Breast Epithelial Cells

To study the mechanisms of ErbB2-induced anoikis resistance of breast cancer cells, we used spontaneously immortalized human non-malignant breast epithelial cells MCF10A (Soule et al., 1990) and their published derivative MCF-ErbB2 generated via infection of MCF10A cells with the wild-type ErbB2-encoding retrovirus and thus stably expressing ErbB2 (Haenssen et al., 2010; M. J. Reginato et al., 2003). We confirmed that similar to what was published by others (Haenssen et al., 2010; M. J. Reginato et al., 2003), detached MCF-ErbB2 cells are noticeably less susceptible to apoptosis (Figure 10a) and more viable (Figure 10b) than the parental MCF10A cells or the control cells MCF10A-vector obtained by the infection of the MCF10A cells with a control retroviral vector. Furthermore, this increased anoikis resistance was paralleled by a strongly increased ability of MCF-ErbB2 cells to survive and form colonies without adhesion to the ECM in soft agar (Figure 10c) (this ability of cancer cells to grow anchorage

independently represents a well-known consequence of their anoikis resistance) (Guadamillas, Cerezo, & del Pozo, 2011; Rosen et al., 1998; K. Rosen et al., 2000).

In an effort to identify ErbB2-dependent mechanisms of anoikis resistance of breast epithelial cells, we found that when detached from the ECM, MCF-ErbB2 cells display much lower levels of the mRNA encoding the pro-apoptotic protein Perp than the parental MCF10A cells or the MCF10A-vector cells (Figure 10d). We further found that detached MCF-ErbB2 cells display a strong Perp downregulation at the protein level compared with MCF10A or MCF10A-vector cells (Figure 10e).

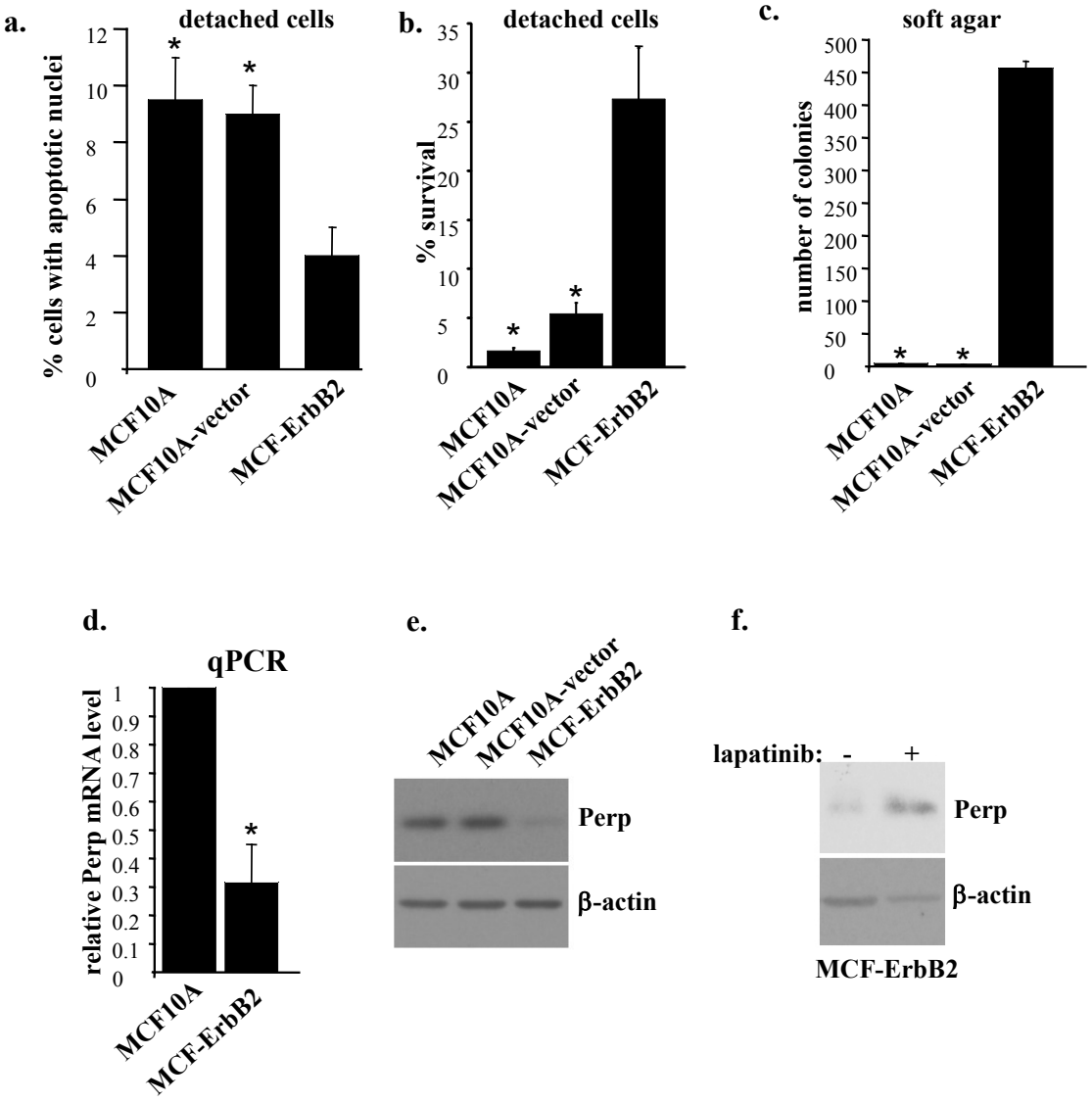

Figure 10. ErbB2 downregulates Perp in detached breast epithelial cells. (a) Non-malignant human breast epithelial cells MCF10A, a control cell line generated by infection of MCF10A cells with a virus obtained by use of an empty retroviral vector (MCF10A-vector) and a variant of MCF10A cells MCF-ErbB2 generated by infection of MCF10A cells with a retrovirus carrying the wild-type ErbB2 were cultured detached from the ECM (in suspension) for 72 h, cell nuclei were stained with Hoechst and % cells with condensed and fragmented nuclei (characteristic features of apoptosis) was determined as the percentage of the cells with such nuclei in a total cell population. (b) MCF10A-vector, MCF-ErbB2 and MCF10A cells were allowed to form colonies in monolayer for 7 days immediately or after being detached for 72 h. % survival was

calculated as a ratio of the number of colonies formed by the cells plated in monolayer after being detached to that formed by the cells plated in a monolayer immediately after transfection × 100%. (c) MCF10A, MCF10A-vector and MCF-ErbB2 cells were allowed to form colonies in monolayer culture or in soft agar for 7–10 days. % colonies were calculated as a ratio between the number of the colonies formed by the cells in soft agar and that formed by the attached cells × 100%. (d) MCF10A and MCF-ErbB2 cells were cultured as in (a) for 2 hand levels of Perp mRNA were determined by quantitative PCR (qPCR). The observed Perp mRNA levels were normalized by the levels of 18S rRNA which were also determined by qPCR. The resulting levels of Perp mRNA in MCF10A cells were arbitrarily designated as 1.0. (e) MCF10A, MCF10A-vector and MCF-ErbB2 cells were cultured detached from the ECM for 3 h and levels of Perp were determined in these cells by western blot. β-Actin served as a loading control. (f) MCF-ErbB2 cells were cultured in suspension for 72 h, in the absence (−) or in the presence (+) of 1 μM lapatinib and assayed as in (e). GAPDH was used as a loading control. Results in (a) represent the average of two independent experiments plus the s.d. Results in (b, c) represent the average of the triplicates plus the s.d. Results in (d) represent the average of three independent experiment plus the s.d. * indicates that P-value was less than 0.05.

Perp is a component of desmosomal multiprotein complexes involved in cell-cell adhesion required to maintain epithelial integrity (Bektas & Rubenstein, 2009; Ihrie et al., 2005). It is a 21 kD tetraspan membrane protein that belongs to Peripheral Myelin Protein (PMP)-22 family of membrane proteins (Attardi et al., 2000) that are known to regulate cell growth (Naef & Suter, 1998). Perp is a transcriptional target of p53-related transcription factor p63 which is also known for maintaining tissue homeostasis (Ihrie et al., 2005). Loss of Perp or p63 are associated with similar epithelial disorders found in mice (Ihrie et al., 2005).

Perp gene expression is also regulated by p53 transcription factor (Reczek, Flores, Tsay, Attardi, & Jacks, 2003). It has been shown to mediate p53-dependent apoptosis through poorly understood mechanism(s) (Ihrie & Attardi, 2004). In addition, Perp is also capable of inducing cell death in a p53-independent manner Whether or not

the ability of Perp to kill cells is related to its involvement in cell-to-cell contacts is not known.

The human Perp gene is located on chromosome 6q24 and loss of heterozygosity for genes located on this chromosome is associated with human ovarian, breast, cervical cancers and melanoma (Millikin, Meese, Vogelstein, Witkowski, & Trent, 1991). Moreover, Perp is often lost in human breast carcinoma cells (Dusek et al., 2012). In addition, loss of the Perp gene promoted mammary tumourigenesis in mice lacking p53 tumour suppressor gene (Dusek et al., 2012) whereas exogenous expression of Perp suppressed such tumour growth (K. Chen, Luo, Li, Liu, & Zhao, 2011).

We further found that treatment of MCF-ErbB2 cells with lapatinib, a small-molecule ErbB2 and EGFR kinase inhibitor (Rusnak et al., 2001) used for treatment of ErbB2-positive advanced breast cancers (N. A. O'Brien et al., 2010), upregulated Perp (Figure 10f). Thus, ErbB2 downregulates Perp in breast epithelial cells.

3.1.2 ErbB2 antagonists upregulate Perp in human breast and ovarian carcinoma cells

In an effort to test whether ErbB2-induced Perp downregulation is relevant to human breast cancer, we found that lapatinib significantly upregulates Perp in detached ErbB2-positive BT-474 human breast cancer cells (N. A. O'Brien et al., 2010) at the protein (Figure 11a, left) and mRNA (Figure 11a, right) levels. Lapatinib also upregulated Perp in detached ErbB2-positive AU-565 human breast tumour cells (Subik et al., 2010) (Figure 11b). To confirm by a genetic approach that ErbB2 blocks Perp expression in these cells, we ablated ErbB2 in both cell lines by two different ErbB2-directed small interfering RNAs (siRNAs) (Figures 11c and e). We found that ErbB2 loss

strongly upregulates Perp in these cells (Figures 11d and f). The effect of lapatinib on Perp was not unique to the indicated cells as we observed a similar effect in ErbB2-positive human breast cancer cells HCC1419 (N. A. O'Brien et al., 2010) and SKBR-3 (N. A. O'Brien et al., 2010) (Figures 11g and h). Furthermore, lapatinib upregulated Perp in ErbB2-positive human ovarian carcinoma cells IGROV-1 (Wilken, Webster, & Maihle, 2010) (Figure 11i). These data are consistent with a scenario in which ErbB2 downregulates Perp in human breast and ovarian cancer cells.

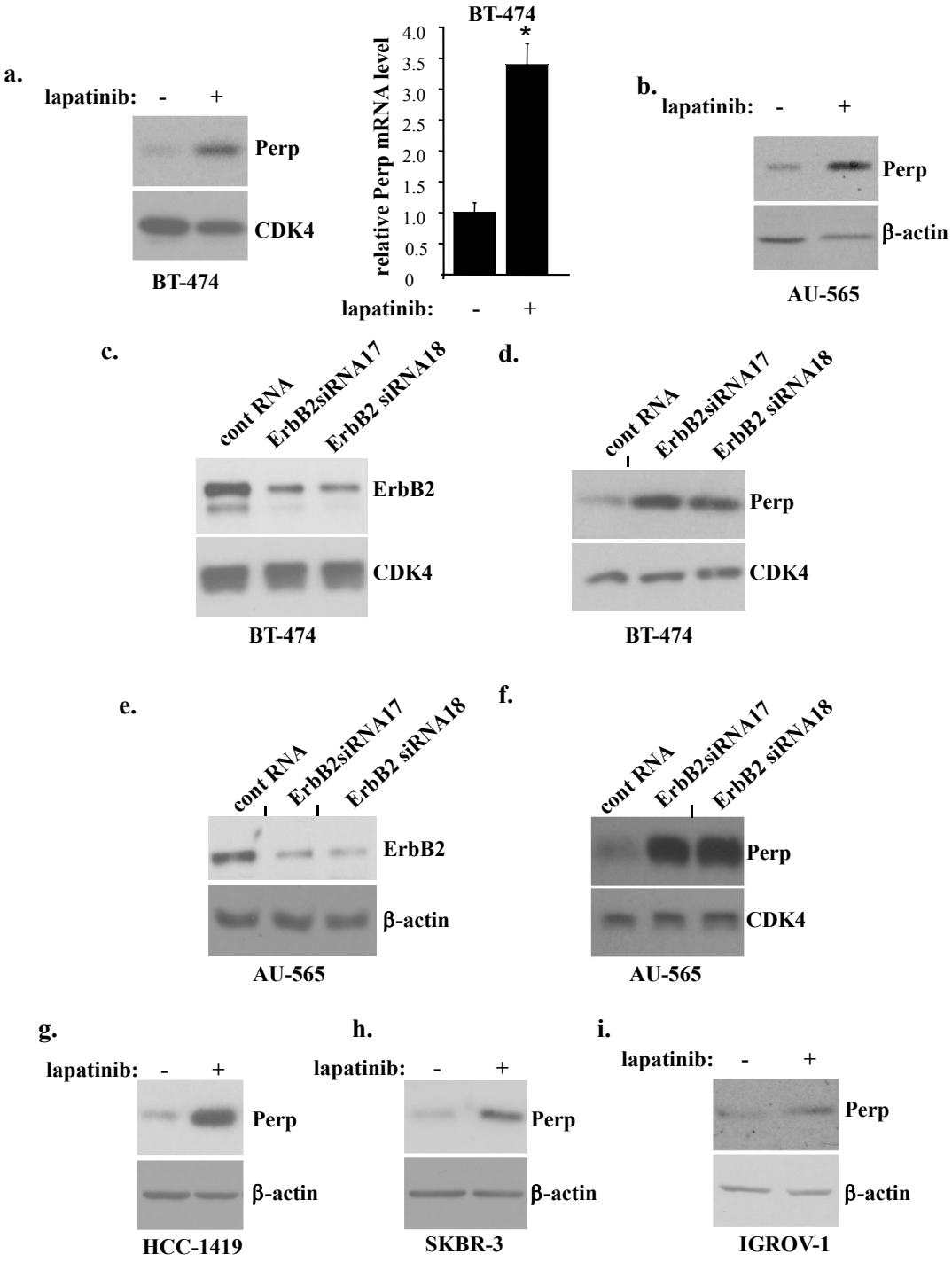

Figure 11. ErbB2 antagonists upregulate Perp in detached human breast carcinoma cells. (a, left) Human breast carcinoma cells BT-474 were cultured detached from the ECM for 48 h in the absence (-) or in the presence (+) of 1 μM lapatinib and assayed for

Perp expression by western blot. (a, right) BT-474 cells were cultured as in (a, left) and levels of Perp mRNA were determined by quantitative PCR (qPCR). The observed Perp mRNA levels were normalized by the levels of 18S rRNA which were also determined by qPCR. The resulting levels of Perp mRNA in untreated BT474 cell were arbitrarily designated as 1.0. (b) Human breast carcinoma cells AU-565 were cultured in suspension for 48 h in the absence (−) or in the presence (+) of 1 μM lapatinib and assayed for Perp expression by western blot. (c) BT-474 were transfected with 100 nM control RNA (contRNA) or ErbB2-specific siRNA (ErbB2 siRNA) 17 or 18, cultured detached from the ECM for 3 h and assayed for ErbB2 expression by western blot. (d) BT-474 cells treated as in (c) were assayed for Perp expression by western blot. (e, f) Human breast carcinoma cells AU-565 were assayed for ErbB2 and Perp expression as in as in (c, d), respectively. (g, h) Human breast carcinoma cells HCC1419 (g) and SKBR-3 (h) were cultured in suspension for 48 h (g) and 72 h (h) in the absence (−) or in the presence (+) of 1 μM lapatinib and assayed for Perp expression by western blot. (i) Human ovarian carcinoma cells IGROV-1 were cultured in suspension for 24 h in the absence (−) or in the presence (+) of 1 μM lapatinib and assayed for Perp expression by western blot. CDK4 served as a loading control in (a, c, d, f) and β-actin served as a loading control in (b, e, g–i).

3.1.3 Detachment-induced Perp upregulation contributes to anoikis of the non-malignant breast epithelial cells.

We further investigated the role of Perp in anoikis of the non- malignant breast epithelial cells. We noticed that detachment of MCF10A cells strongly upregulates Perp and that ErbB2 blocks this upregulation (Figure 12a). Perp upregulation in MCF10A cells was observed at 3 h of detachment (Figure 12a), a time point that preceded the onset of anoikis in these cells (Figure 12b). To test whether Perp contributes to detachment-induced apoptosis of MCF10A cells, we ablated Perp in these cells by two separate Perp-specific siRNAs (Figure 12c). We found that Perp loss significantly reduces anoikis of MCF10A cells (Figure 12d). Thus, detachment-induced Perp upregulation contributes to anoikis of the non-malignant breast epithelial cells.

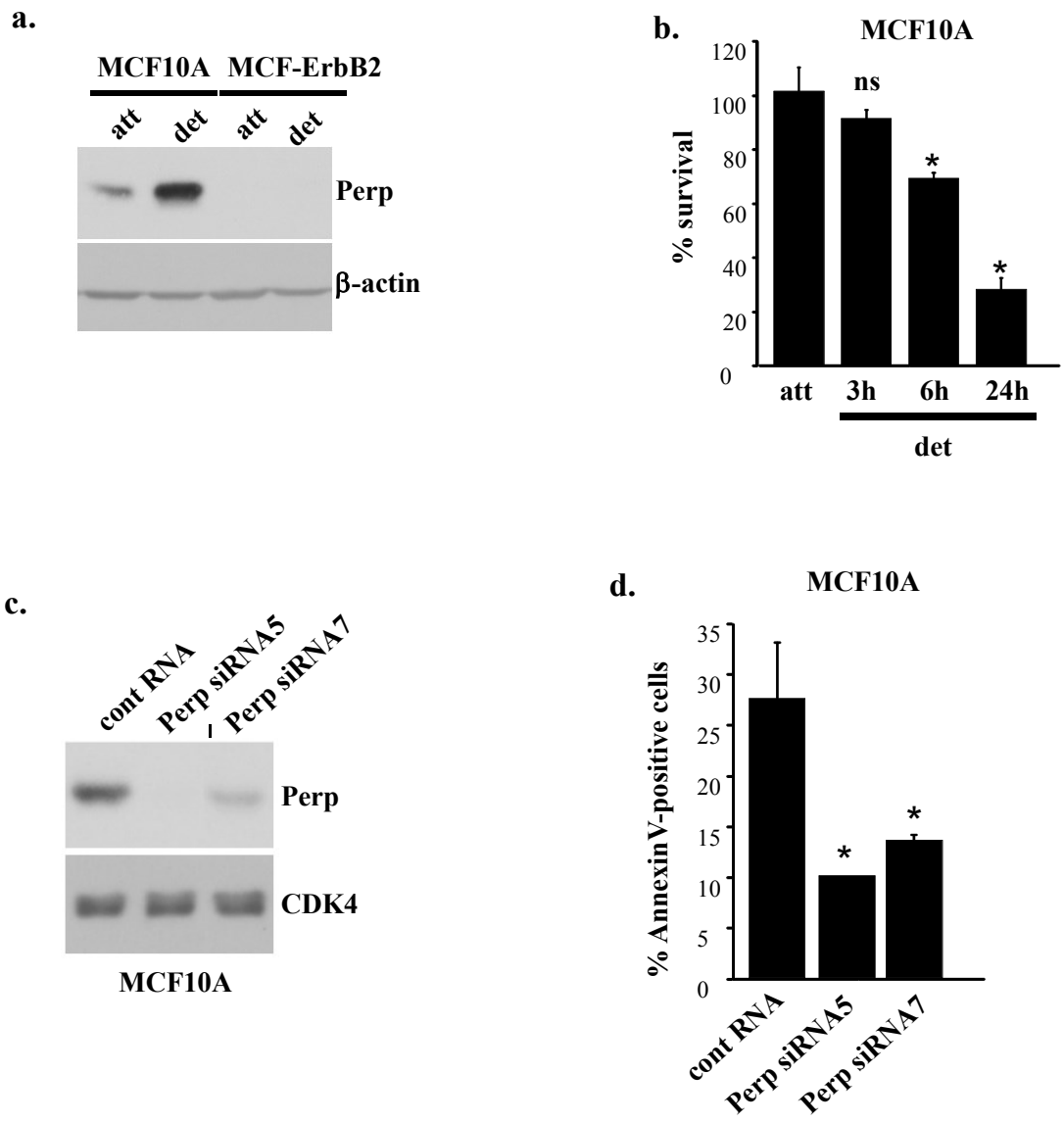

Figure 12. Detachment-induced upregulation of Perp is required for anoikis of non-malignant breast epithelial cells. (a) Indicated cells were cultured attached to the ECM (att) or detached from the ECM (det) for 3 h and assayed for Perp expression by western blot. (b) MCF10A cells were allowed to form colonies in monolayer for 7 days immediately or after being detached for the indicated times. % survival was calculated as a ratio of the number of colonies formed by the cells plated in monolayer after being detached to that formed by the cells plated in monolayer immediately ×100%. (c) MCF10A cells were transfected with 100nM control RNA (contRNA) or Perp-specific siRNA (Perp siRNAs) 5 or 7 and assayed for Perp expression by western blot. (d)

MCF10A cells were treated as in (c), cultured detached from the ECM for 24 h, stained with propidium iodide (PI) and assayed for Annexin V binding (a characteristic feature of dying cells) by flow cytometry. % Annexin V-positive cells was calculated as the sum of the percentage of Annexin V-positive/PI-negative cells and that of Annexin V-positive/ PI-positive cells in a total cell population. The data represent the average of the triplicates plus the s.d. (b) and two independent experiments plus the s.d. (d). CDK4 was used as a loading control in (a, c). * indicates that P-value was less than 0.05. NS, not significant.

3.1.4 ErbB2-induced Perp downregulation is required for anoikis resistance of breast epithelial cells.

To test whether ErbB2-induced Perp downregulation contributes to anoikis resistance of breast epithelial cells we reversed this downregulation. To this end, we transiently expressed ectopic green fluorescent protein (GFP)-tagged-Perp in MCF-ErbB2 cells (Figure 13a). We observed that exogenous Perp significantly increased apoptosis of detached MCF-ErbB2 cells (Figure 13b).

To test the role of Perp in anoikis resistance of ErbB2- overexpressing breast epithelial cells by a complementary approach, we infected MCF-ErbB2 cells with a Perp-encoding lentivirus and generated a variant of these cells stably expressing ectopic Perp (MCF-ErbB2-Perp) (Figure 13c). Importantly, when detached from the ECM, these cells expressed Perp at levels that were significantly higher than those in the control cells (MCF-ErbB2-cont) infected with the lentiviral vector alone but did not exceed those in the detached parental MCF10A cells (Figure 13c). Hence, Perp was produced by MCF-ErbB2-Perp cells at physiological levels. We found that MCF-ErbB2-Perp cells displayed a strongly reduced ability to form colonies without adhesion to the ECM in soft agar compared with the control cells (Figure 13d). Furthermore, this decreased ability to grow without adhesion to the ECM over an extended period of time was paralleled by a

significantly increased apoptosis of MCF-ErbB2- Perp compared with the control cells at various times following detachment from the ECM (Figures 13e-g). Thus, ErbB2-induced Perp loss is required for ErbB2-dependent anoikis resistance of breast epithelial cells.

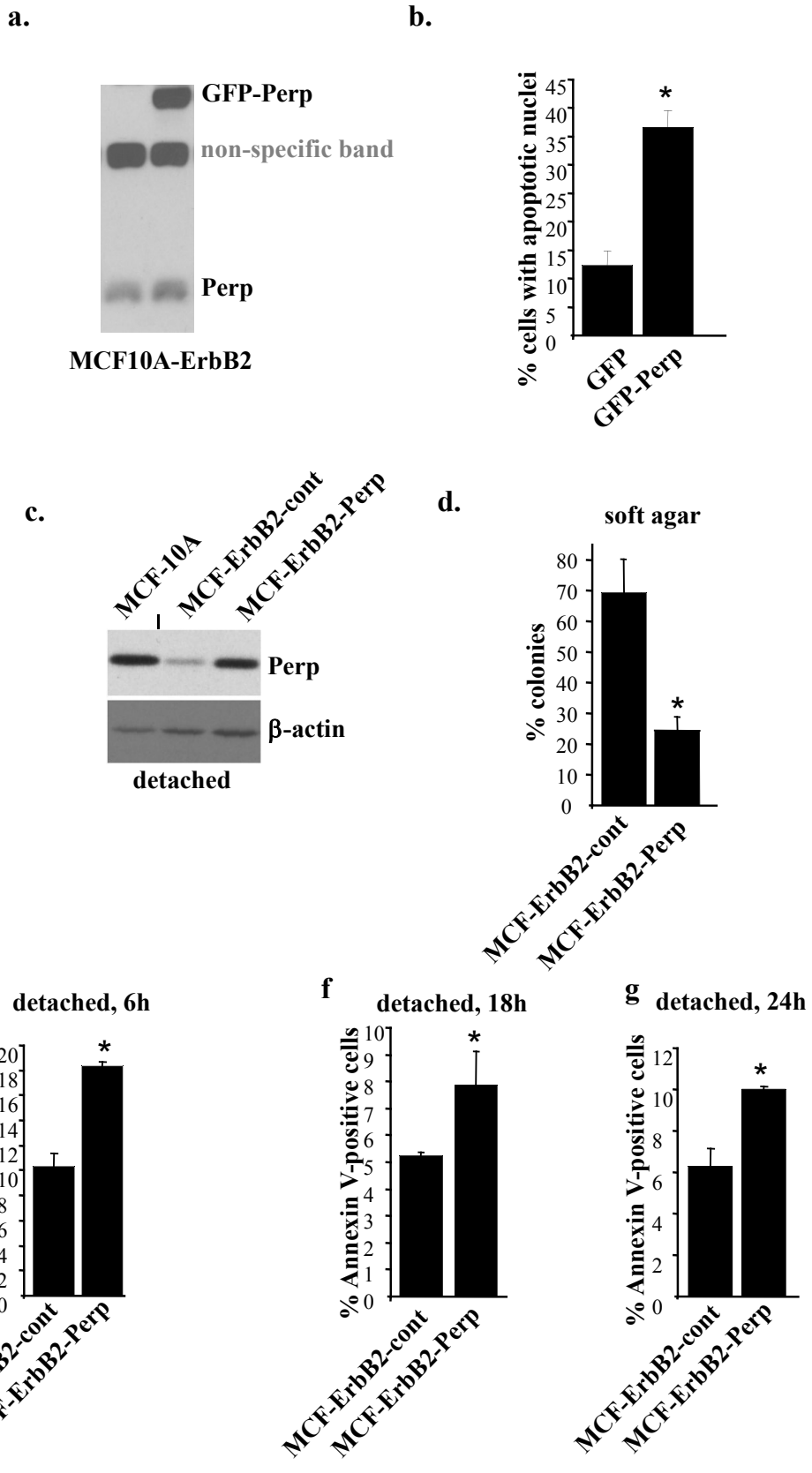
a.
GFP-Perp
non-specific band
Perp
MCF10A-ErbB2
b.
% cells with apoptotic nuclei
45
40
35
30
25
20
15
10
5
0
*
GFP
GFP-Perp
c.
MCF-10A
MCF-ErbB2-cont
MCF-ErbB2-Perp
Perp
β-actin
detached
d.
soft agar
% colonies
80
70
60
50
40
30
20
10
0
*
MCF-ErbB2-cont
MCF-ErbB2-Perp
e.
detached, 6h
% Annexin V-positive cells
20
18
16
14
12
10
8
6
4
2
0
*
MCF-ErbB2-cont
MCF-ErbB2-Perp
f
detached, 18h
% Annexin V-positive cells
10
9
8
7
6
5
4
3
2
1
0
*
MCF-ErbB2-cont
MCF-ErbB2-Perp
g
detached, 24h
% Annexin V-positive cells
12
10
8
6
4
2
0
*
MCF-ErbB2-cont
MCF-ErbB2-Perp

Figure 13. Downregulation of Perp is required for ErbB2-induced anoikis resistance of breast epithelial cells. (a) MCF-ErbB2 cells were transiently transfected with a control vector encoding green fluorescent protein (GFP) or an expression vector encoding GFP- fused Perp (GFP-Perp) and assayed for Perp expression by western blot. Position of the endogenous Perp (Perp), exogenous GFP-Perp (GFP-Perp) and that of a nonspecific band on the gel is indicated. (b) Cells treated as in (a) were cultured detached from the ECM for 48h, cell nuclei were stained with Hoechst and % cells with condensed and fragmented nuclei (characteristic features of apoptosis) was determined as the percentage of the cells with such nuclei in a population of GFP-positive cells. The data in (b) represent the average of the triplicates plus the s.d. (c) MCF10A cells, a variant of MCF-ErbB2 cells generated by infection of these cells with a control lentivirus obtained by use of a control pLex-MCS expression vector (MCF-ErbB2-cont) or a variant of MCF-ErbB2 cells stably expressing ectopic Perp (MCF-ErbB2-Perp) generated by infection of MCF-ErbB2 cells with a pLex-MCS expression vector carrying the Perp cDNA were cultured detached from the ECM for 3 h and assayed for Perp expression by western blot. β-Actin served as a loading control. (d) Indicated cell lines were allowed to form colonies for 7–10 days in monolayer or soft agar. % colonies were calculated as a ratio between the number of the colonies formed by the cells in soft agar and that formed by the attached cells × 100%. The data represent the average of four independent experiments plus the s.e. (e–g) Indicated cell lines were cultured detached from the ECM for 6 (e), 18 (f) and 24 h (g), stained with propidium iodide (PI) and assayed for Annexin V binding (a characteristic feature of dying cells) by flow cytometry. % Annexin V-positive cells was calculated as the sum of the percentage of Annexin V-positive/PI- negative cells and that of Annexin V-positive/PI-positive cells in a total cell population. The data represent the average of the triplicates plus the s.e. * indicates that P-value was less than 0.05.

3.1.5 ErbB2-induced downregulation of Perp in breast epithelial cells is Mek-dependent.

Mitogen-activated protein kinase and PI 3-kinase represent major effectors of ErbB2 signaling (Yarden & Sliwkowski, 2001). We therefore tested whether these enzymes mediate ErbB2-dependent Perp loss in breast epithelial cells. ErbB2 triggers mitogen-activated protein kinase signaling by activating a small GTPase Ras, which in turn activates protein kinases Raf (Yarden & Sliwkowski, 2001). Raf kinases phosphorylate and thus activate protein kinases Mek1/2. Mek kinases further

phosphorylate and activate the Erk 1 and 2 protein kinases (Kolch, 2000; Santen et al., 2002). Erk1/2 subsequently phosphorylate and alter the activity of numerous cellular proteins.

We found that treatment with a widely used specific Mek inhibitor PD98059 significantly blocked phosphorylation of Erk1 and 2 (we mainly detected phospho- Erk2 in our model system) (levels of phospho-Erk1 or 2 are typically measured in cells to assess Mek activity (Kirill Rosen et al., 2000) (Figure 14a) and strongly upregulated Perp in detached MCF-ErbB2 cells (Figure 14b). Thus, ErbB2 downregulates Perp in breast epithelial cells in a Mek-dependent manner.

We further tested whether activation of Mek is sufficient for Perp downregulation in breast epithelial cells. To this end, we used a published (Haenssen et al., 2010) derivative of MCF10A cells stably expressing active mutant form of Mek kinase generated via infection of MCF10A cells with a retrovirus encoding a constitutively active mutant form of Mek kinase in which serine (S) residues at positions 222 and 226 were substituted by aspartate (D) (Voisin et al., 2008). Aspartate residues mimic the phosphorylated serine residues at those positions and maintains the protein in its constitutively active form (Léger, Kempf, Lee, & Brandt, 1997). We found that detached MCF-MekDD cells display much lower Perp levels than the parental MCF10A or MCF10A-vector cells (Figure 14c). Hence, activation of Mek is sufficient for Perp loss in detached breast epithelial cells.

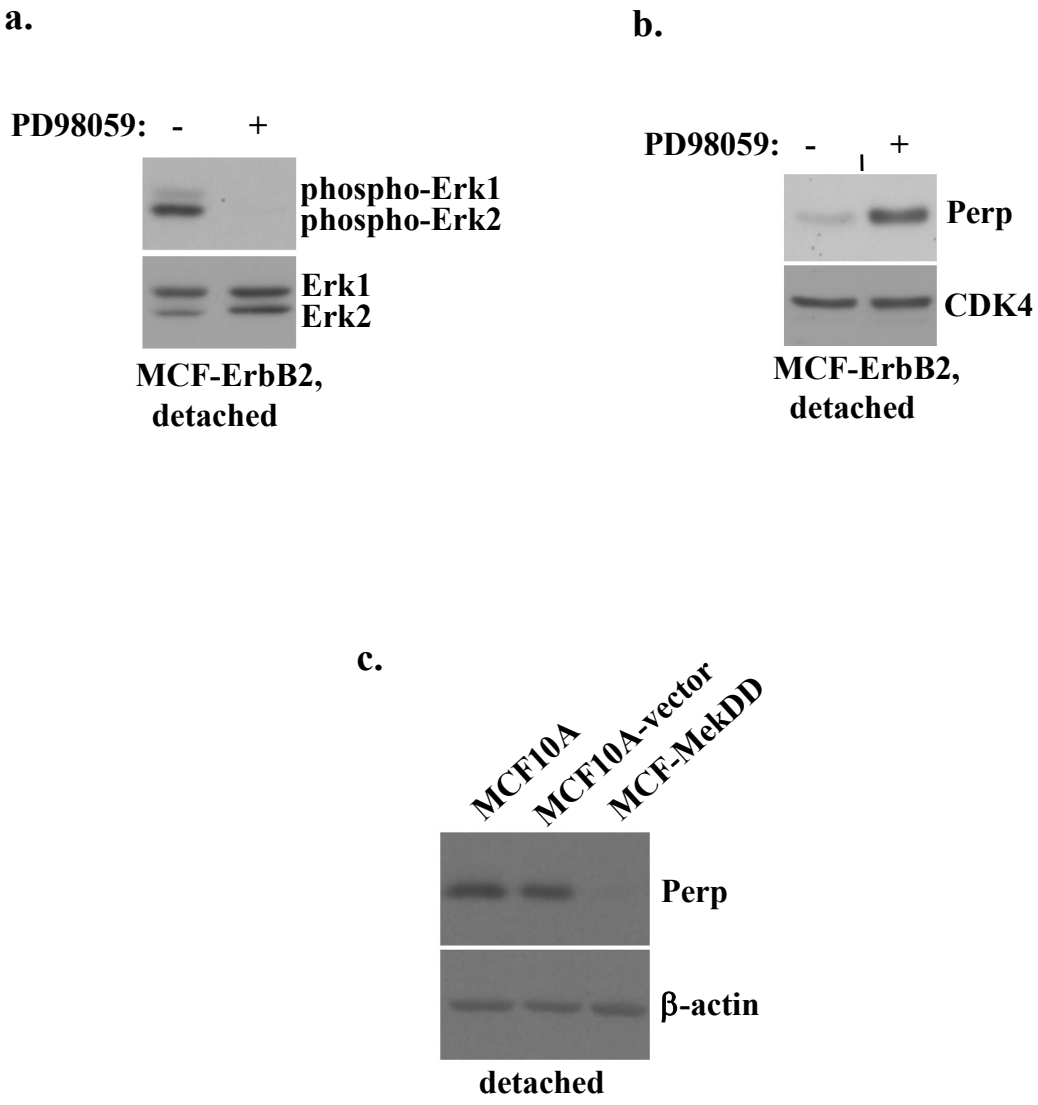

Figure 14. ErbB2-induced Perp downregulation occurs in a Mek- dependent manner. (a) MCF-ErbB2 cells were cultured for 5 h detached from the ECM in the presence of DMSO (-) or 25 μM PD98059 (+) and assayed for phospho-Erk1/2 expression by western blot. The membrane was re-probed with an anti-Erk1/2 antibody. (b) MCF-ErbB2 cells were cultured as in (a) and assayed for Perp expression by western blot. The membrane was re-probed with an anti-CDK4 antibody. (c) MCF10A, MCF10A-vector cells and a variant of MCF10A cells obtained by infection of these cells with a retrovirus carrying constitutively active Mek2 mutant (MCF-MekDD) were cultured detached from the ECM for 3 h and assayed for Perp expression by western blot. The membrane was re-probed with an anti-β-actin antibody.

We also observed that treatment of detached MCF-ErbB2 cells with a PI 3-kinase inhibitor LY294002 (Vlahos, Matter, Hui, & Brown, 1994) blocked phosphorylation of a protein kinase Akt, a known PI3K target (Figures 15a-c) (levels of phospho-Akt are typically determined in cells to measure PI 3-kinase activity (Rosen et al., 1998), but did not upregulate Perp in these cells (Figures 15 d-f). Hence, ErbB2 downregulates Perp in the indicated cells in a PI 3-kinase-independent manner. In summary, our data indicate that Mek activation is necessary and sufficient for ErbB2-induced Perp loss in breast epithelial cells.

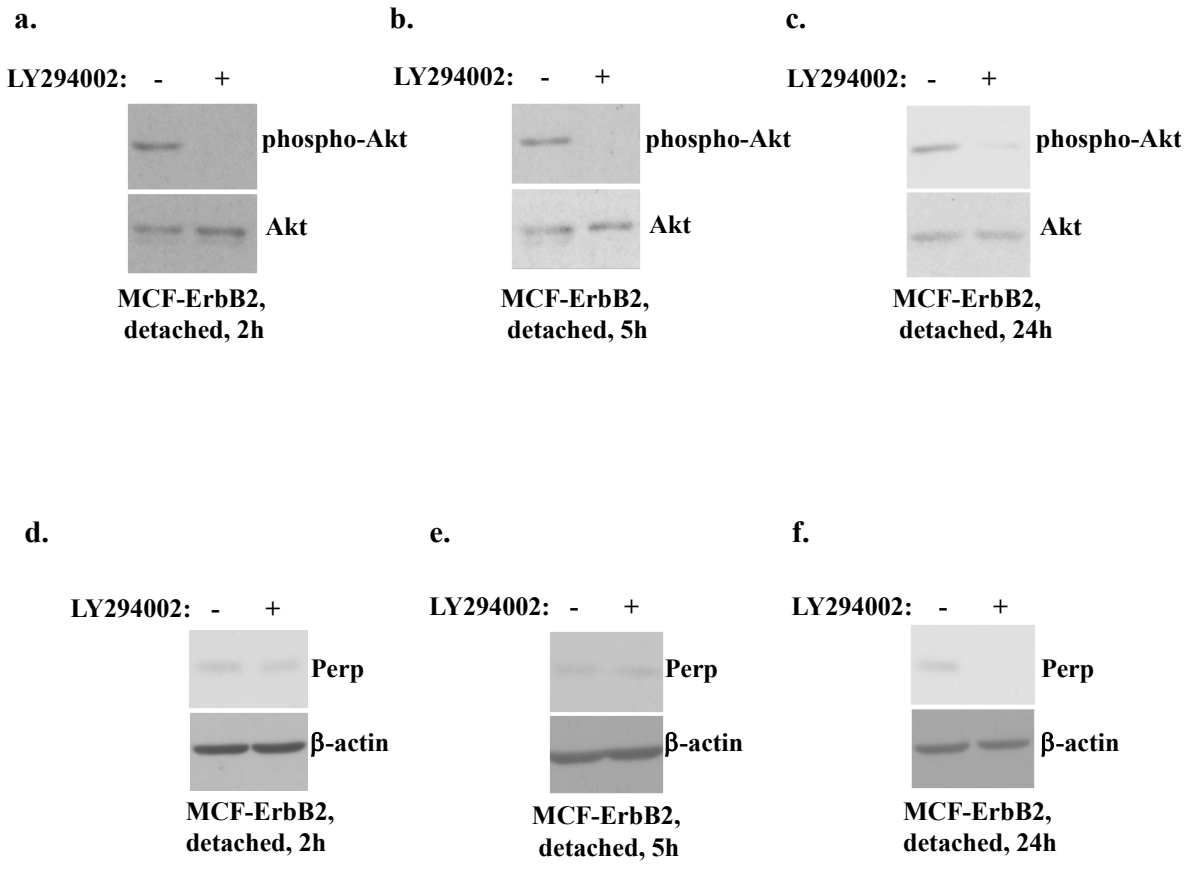

Figure 15. ErbB2-induced Perp downregulation cannot be blocked by a PI 3-kinase inhibitor. (a-c) MCF-ErbB2 cells were cultured for 2 h (a), 5 h (b) or 24 h (c) detached

from the ECM in the presence of DMSO (-) or 40 μM LY294002 (+) and assayed for phospho-Akt expression by western blot. The membrane was re-probed with an anti-Akt antibody. (d-f) MCF-ErbB2 cells were cultured as in (a-c) and assayed for Perp expression by western blot. The membrane was re-probed with an anti-β-actin antibody.

3.1.6 ErbB2 downregulates Perp in breast epithelial cells by blocking detachment-induced EGFR loss

In an effort to further understand the mechanisms by which ErbB2 downregulates Perp, we reasoned that one mitogen-activated protein kinase-dependent mechanism that contributes to ErbB2-induced inhibition of anoikis is driven by ErbB2-driven upregulation of EGFR in breast epithelial cells (Grassian, Schafer, et al., 2011). It was found that detachment of MCF10A cells downregulates EGFR and that this contributes to their anoikis (Grassian, Schafer, et al., 2011; M. J. Reginato et al., 2003). It was also observed that detachment-induced EGFR downregulation is driven by the lysosomal degradation of this receptor (Grassian, Schafer, et al., 2011). Such degradation is mediated by the E3-ubiquitin ligase c-Cbl that targets EGFR to the lysosomes (Roepstorff, Grøvdal, Grandal, Lerdrup, & van Deurs, 2008). It was shown that ErbB2-induced Erk activation in detached MCF10A cells promotes increased expression of a signaling protein Sprouty-2 which binds and inactivates c-Cbl and thus suppresses c-Cbl-dependent EGFR degradation (Grassian, Coloff, & Brugge, 2011). Increased EGFR expression or activity is in turn well known to block anoikis of MCF10A cells and that of epithelial cells derived from other organs (Jost, Huggett, Kari, & Rodeck, 2001; M. J. Reginato et al., 2003; Rosen et al., 2001; Schulze, Lehmann, Jefferies, McMahon, & Downward, 2001).

We verified that detachment of MCF10A cells results in a strong EGFR downregulation (Figure 16a). Furthermore, we observed that ectopic ErbB2 significantly upregulates EGFR in detached MCF10A cells (Figure 16b). We also found that Mek inhibitor PD98059 noticeably downregulates EGFR in detached MCF-ErbB2 cells (Figure 16c), whereas activated Mek upregulates EGFR in detached MCF10A cells (Figure 16d). Thus, we confirmed that detachment of breast epithelial cells downregulates EGFR while ErbB2 prevents this downregulation in a Mek-dependent manner (Grassian, Schafer, et al., 2011).

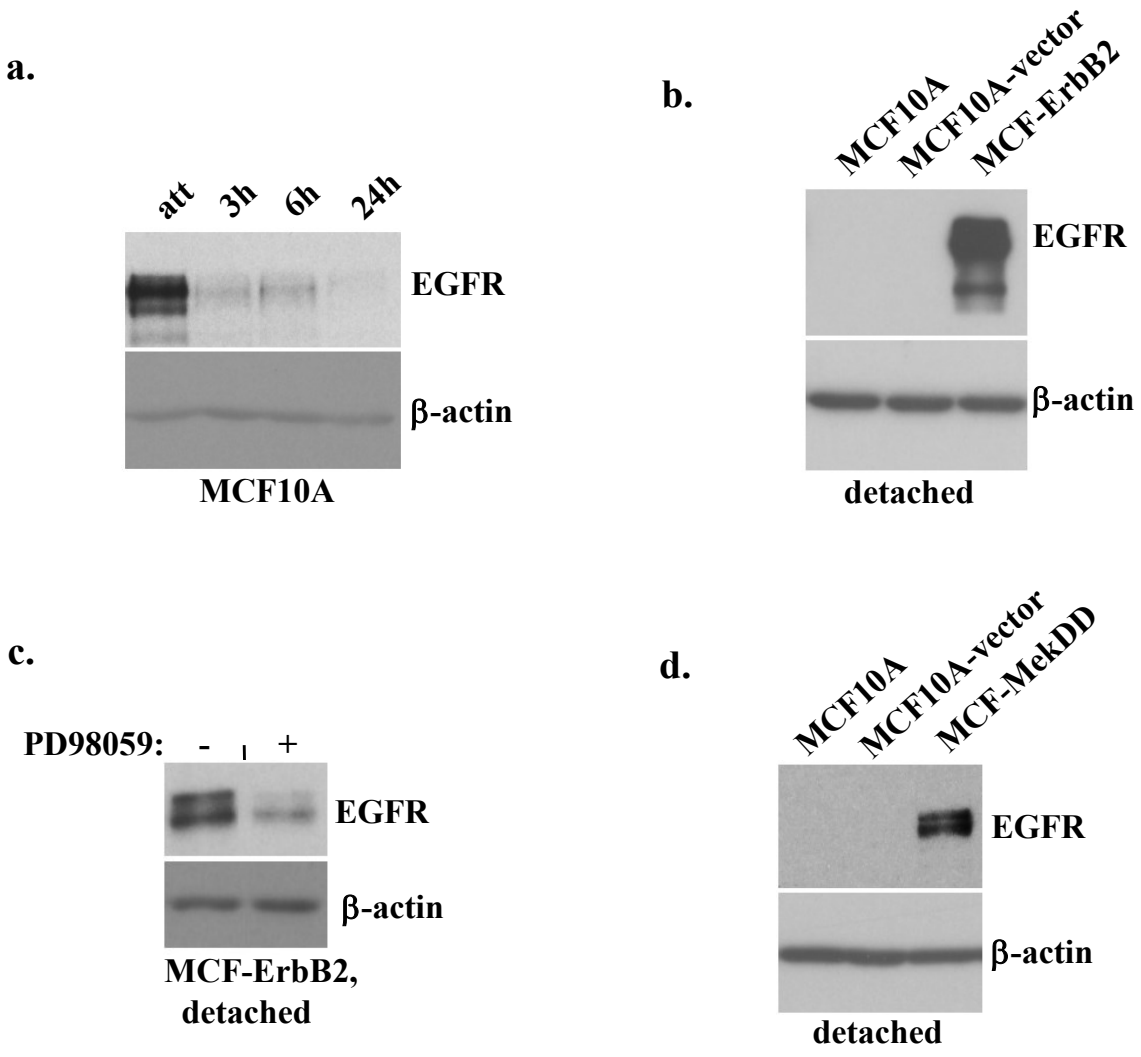

Figure 16. ErbB2 blocks detachment-induced EGFR loss in a Mek- dependent manner. (a) MCF10A cells were cultured attached to the ECM (att) or detached from the ECM (det) for the indicated times and assayed for EGFR expression by western blot. (b) MCF10A, MCF10A- vector and MCF-ErbB2 cells were cultured detached from the ECM for 3 h and assayed for EGFR expression by western blot. (c) MCF-ErbB2 cells were cultured for 24 h detached from the ECM in the presence of DMSO (−) or 25 μM PD98059 (+) and assayed for Perp expression by western blot. (d) MCF10A, MCF10A-vector and MCF-MekDD cells were cultured detached from the ECM for 3h and assayed for EGFR expression by western blot. β-Actin was used as a loading control in (a–d). When lanes were removed from western blot images, and separate parts of an image were joined together, a short vertical black line was used to indicate where the image was cut.

We further tested whether ErbB2-induced Perp downregulation is EGFR dependent. To this end, we ablated EGFR in MCF-ErbB2 cells by two separate EGFR-directed siRNAs (Figure 17a). We found that EGFR loss significantly upregulates Perp in detached MCF- ErbB2 cells (Figure 17b). Hence, EGFR is necessary for ErbB2-dependent Perp downregulation. We further investigated whether EGFR inactivation is sufficient for Perp upregulation in the non-malignant breast epithelial cells. To achieve this, we took advantage of the fact that MCF10A cells are normally cultured in the medium that includes an EGFR ligand EGF (Haenssen et al., 2010; M. J. Reginato et al., 2003). We found that attached MCF10A cells cultured in the medium to which EGF was not added display much higher Perp levels than MCF10A cells cultured in the standard EGF-containing medium (Figure 17c). Furthermore, we observed that Perp was not upregulated when the cells were cultured in the absence of serum (which is normally present in the medium used for culturing these cells (Haenssen et al., 2010; M. J. Reginato et al., 2003) but in the presence of EGF (Figure 17c). In contrast, EGF ablation strongly upregulated Perp in the cells cultured in the serum-free medium. Hence, EGFR inactivation (caused by EGF ablation in our experiments) is sufficient for Perp upregulation in the non- malignant breast epithelial cells whereas no other factors present in the serum are capable of downregulating Perp. Collectively, our data indicate that ErbB2 downregulates Perp in detached breast epithelial cells by blocking detachment-induced EGFR loss.

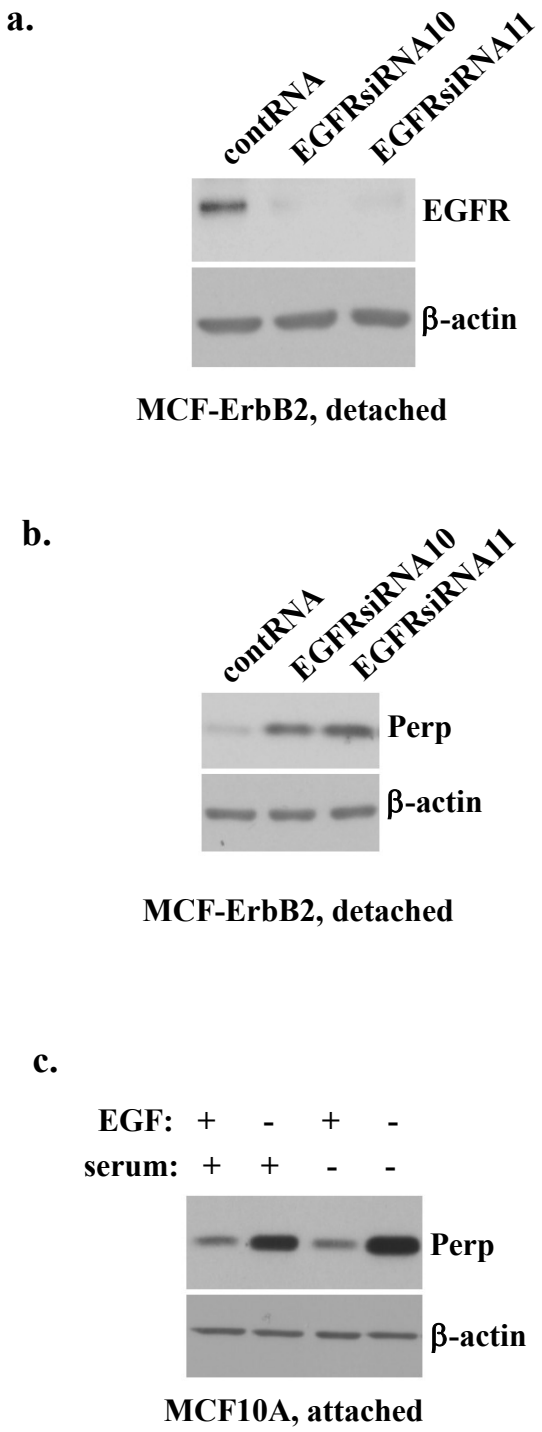

Figure 17. ErbB2-dependent Perp downregulation occurs in an EGFR-dependent manner. (a) MCF-ErbB2 cells were transfected with 100 nM control RNA (contRNA) or EGFR-specific siRNA (EGFR siRNAs) 10 or 11, cultured detached from the ECM for 3

h and assayed for EGFR expression by western blot. (b) MCF-ErbB2 cells treated as in (a) were cultured detached from the ECM for 3 h and assayed for Perp expression by western blot. (c) MCF10A cells were cultured attached to the ECM for 24 h in the regular growth medium that includes serum and EGF (+) or in a medium to which EGF and/or serum were not added (−) and assayed for Perp expression by western blot. β-actin was used as a loading control in (a–c).

Others found that ErbB2 upregulates a regulatory protein Sprouty-2 in a Mek-dependent manner in detached MCF-ErbB2 cells and that it is Sprouty-2 that blocks detachment-induced EGFR degradation in these cells (by neutralizing E3-ubiquitin ligase c-Cbl that targets EGFR to the lysosomes) (Grassian, Schafer, et al., 2011). Thus, we tested whether Sprouty-2 is required for ErbB2-induced Perp loss in these cells. We found that enforced downregulation of Sprouty-2 in MCF- ErbB2 cells by two separate siRNAs (Figures 18a and b) noticeably upregulated Perp (Figures 18c and d).

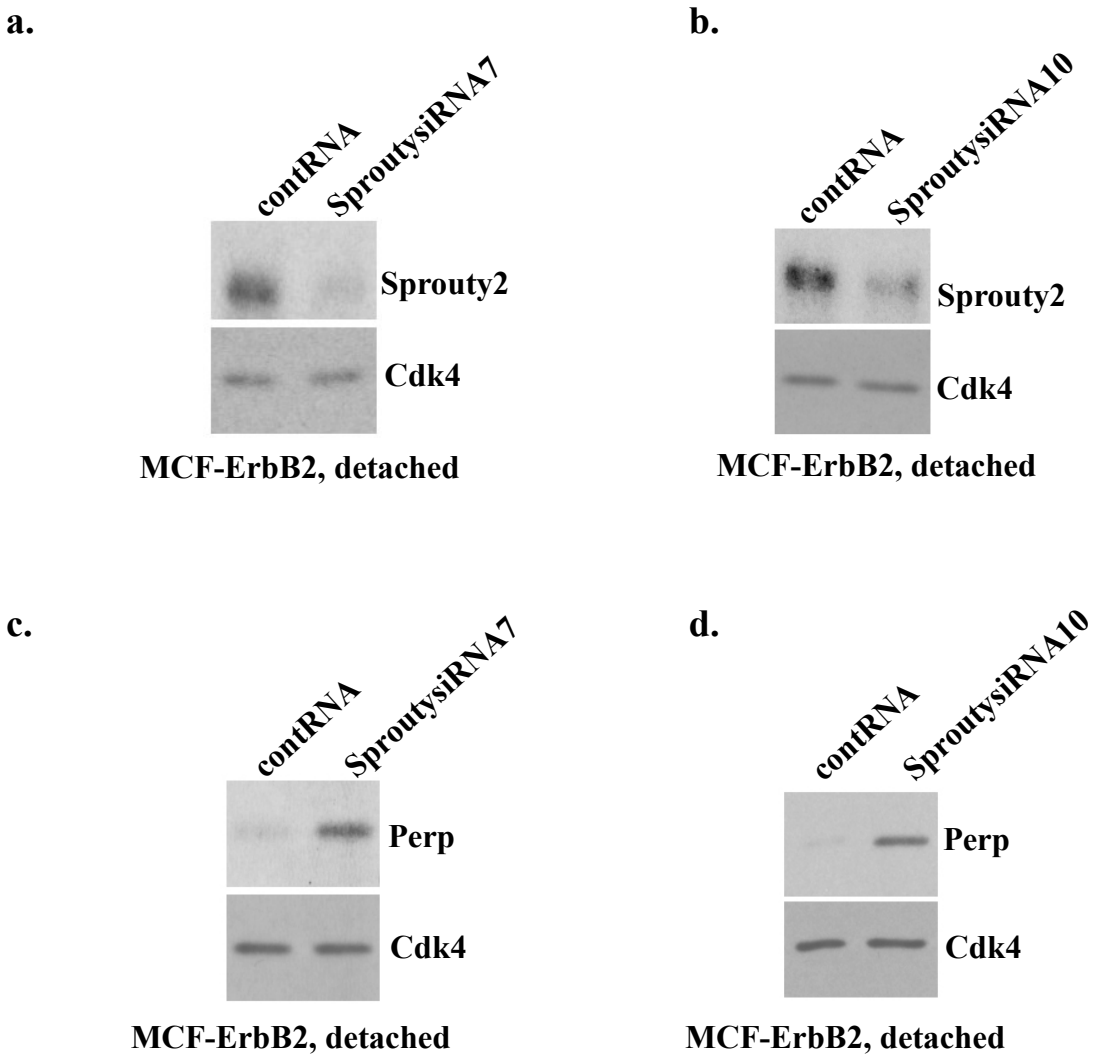

Figure 18. ErbB2-dependent Perp downregulation occurs in a Sprouty-2-dependent manner. MCF-ErbB2 cells were transfected with 100nM control RNA (contRNA) or Sprouty-2-specific siRNA (Sprouty siRNA) 7 (a) or 10 (b), cultured detached from the ECM for 3h and assayed for Sprouty-2 expression by western blot. (c, d) MCF-ErbB2 cells treated as in (a, b), respectively, were cultured detached from the ECM for 24 h and assayed for Perp expression by western blot. CDK4 was used as a loading control.

3.1.7 Summary

In summary, we have identified a novel ErbB2-dependent mechanism of anoikis resistance of breast epithelial cells. This mechanism is mediated by ErbB2-induced activation of Mek, subsequent upregulation of Sprouty-2 and Sprouty-2-dependent inhibition of EGFR loss caused by detachment of the indicated cells from the ECM (Grassian, Schafer, et al., 2011). Once this loss is prevented, EGFR promotes Perp downregulation in detached cells. Perp loss in turn contributes to anoikis resistance of such cells.

3.2 ErbB2-driven downregulation of a transcription factor Irf6 in breast epithelial cells is required for their three-dimensional growth.

The data presented below represent the results of our continued investigation on ErbB2-induced mechanisms that block anoikis of breast cancer cells. The text in this chapter is presented verbatim of the submitted manuscript (Iman Aftab Khan, 2018) with the exception of those cases when the text was modified according to the Dalhousie University Thesis guidelines. See Appendix A for author contributions.

3.2.1 ErbB2 downregulates Irf6 in breast epithelial cells.

In an effort to further investigate the mechanisms by which ErbB2 blocks anoikis of breast cancer cells we found that the wild type ErbB2 strongly downregulates Irf6 in detached MCF10A cells both at the mRNA (Figure 19a) and the protein (Figure 19b) level.

Irf6, a member of the Irf transcription factor family (Taniguchi, Ogasawara, Takaoka, & Tanaka, 2001). Members of this family are well known to control interferon and interferon-inducible genes in response to various pathogenic stimuli (Negishi, Taniguchi, & Yanai, 2017). In addition, Irf6 loss of function mutations are widely recognized to cause an autosomal dominant disorder characterized by cleft lip and palate with lip pits referred to as the Van der Woude syndrome (Kohli & Kohli, 2012). Importantly for this study, Irf6 can kill cells by apoptosis (Y. Lin et al., 2016) and is likely an important mediator of breast function as Irf6 is upregulated in the breast during mammary gland regression upon cessation of lactation (such regression likely involves anoikis of breast epithelial cells (J. Wiesen & Z. Werb, 2000). According to a published

immunohistochemical study, Irf6 protein is often downregulated in breast cancer (Uhlen et al., 2005). Moreover, Irf6 was also shown to be downregulated in nasopharyngeal (Xu et al., 2017) and squamous cell (Botti et al., 2011) carcinomas. How Irf6 activity is regulated in cells has not been studied in detail. Irf6 can be phosphorylated at several amino acid residues (Bailey & Hendrix, 2008; Kwa et al., 2014) which is the reason why Irf6 is often detected as a doublet on western blots (Bailey & Hendrix, 2008). Two Irf6 phosphorylation sites were identified and their phosphorylation was shown to be required for Irf6 binding to a protein kinase IRAK1 and for Irf6 transcriptional activity in certain contexts (Kwa et al., 2014). To our knowledge, Irf6 has never been implicated as a mediator of ErbB2 signalling breast tumour cells.

In an effort to test whether ErbB2 downregulates Irf6 human breast cancer we observed that lapatinib, a small molecule ErbB2/EGFR inhibitor used for treatment of ErbB2-positive metastatic breast cancer (Geyer et al., 2006) strongly upregulates Irf6 in detached ErbB2-positive human breast cancer cells BT474, AU-565 and HCC1419 (Subik et al., 2010) (Figure. 19c-e). Moreover, we found that the anti-ErbB2-antibody trastuzumab normally used for ErbB2 positive breast cancer treatment (Slamon et al., 2001) upregulates Irf6 in BT474 cells (Figure 19f) but not in the variant of these cells selected for trastuzumab resistance by prolonged exposure of the cells to trastuzumab in suspension culture (Figure 19g). Thus, ErbB2 downregulates Irf6 in human breast cancer cells detached from the ECM.

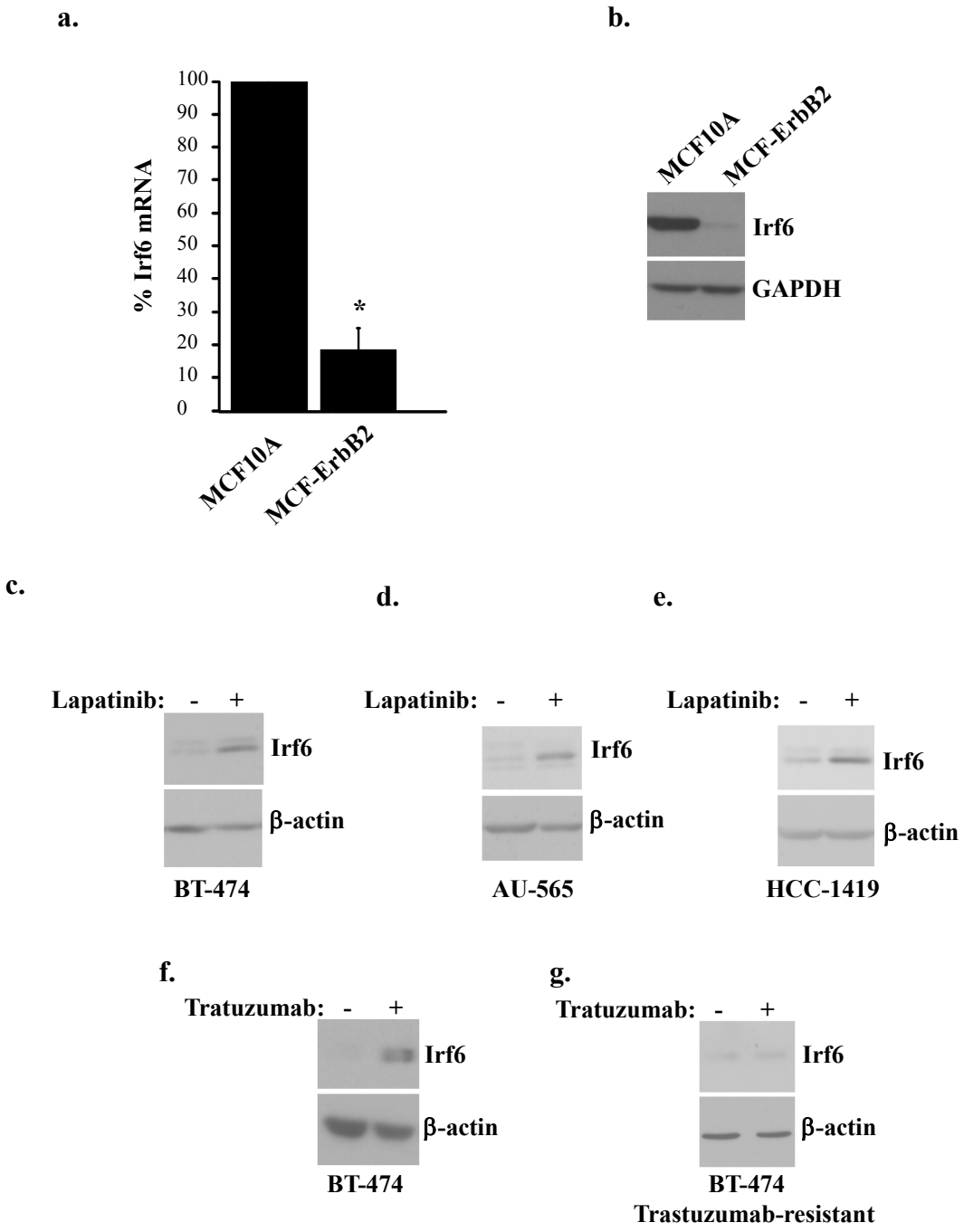

Figure 19. ErbB2 downregulates Irf6 in detached breast epithelial cells. (a) MCF10A and a variant of MCF10Acells MCF-ErbB2 generated by infection of MCF10A cells with a retrovirus carrying the wild type ErbB2 were cultured detached from the ECM (in

suspension) for 2h and Irf6 mRNA levels were analyzed in the cells detached by quantitative PCR (qPCR). Irf6 mRNA levels were normalized by those of 18S rRNA (determined by qPCR). The resulting Irf6 mRNA levels in MCF10A cells were designated as 100%. Results represent the average of two independent experiments plus the S.D. * - p-value is less than 0.05. (b) MCF10A and MCF-ErbB2 cells were cultured as in (a) for 3h and assayed for Irf6 levels by western blot. GAPDH served as a loading control. (c-e) Human breast carcinoma cells BT-474 (c), AU-565 (d) and HCC-1419 (e) were cultured detached from the ECM for 48h in the absence (-) or in the presence (+) of 1 μM lapatinib and assayed for Irf6 expression by western blot. (f, g) BT-474 cells (f) or their trastuzumab-resistant variant were cultured detached from the ECM for 48h in the absence (-) or in the presence (+) of 5 μg/ml trastuzumab and assayed for Irf6 expression by western blot. β-actin was used as a loading control in (c-g).

3.2.2 Detachment-induced Irf6 upregulation contributes to anoikis of non-malignant breast epithelial cells.

We further found that detachment of MCF-10A cells from the ECM upregulates Irf6 (Figure 20a). Moreover, detachment of anoikis-susceptible non-malignant primary human mammary epithelial cells (HMEC) (Dontu, Abdallah, et al., 2003) had a similar effect on Irf6 (Figure 20b) indicating that detachment-induced Irf6 upregulation is not unique to MCF-10A cells (Figure 20a).

To investigate whether Irf6 contributes to anoikis of breast epithelial cells we knocked down Irf6 in MCF10A cells by two different Irf6-specific siRNAs (Figure 20c). We found that enforced Irf6 downregulation increased survival of detached MCF10A cells (Figure 20d) and substantially reduced their apoptosis in suspension culture (Figure 20e). These data indicate that detachment-induced Irf6 upregulation contributes to anoikis of non-malignant breast epithelial cells.

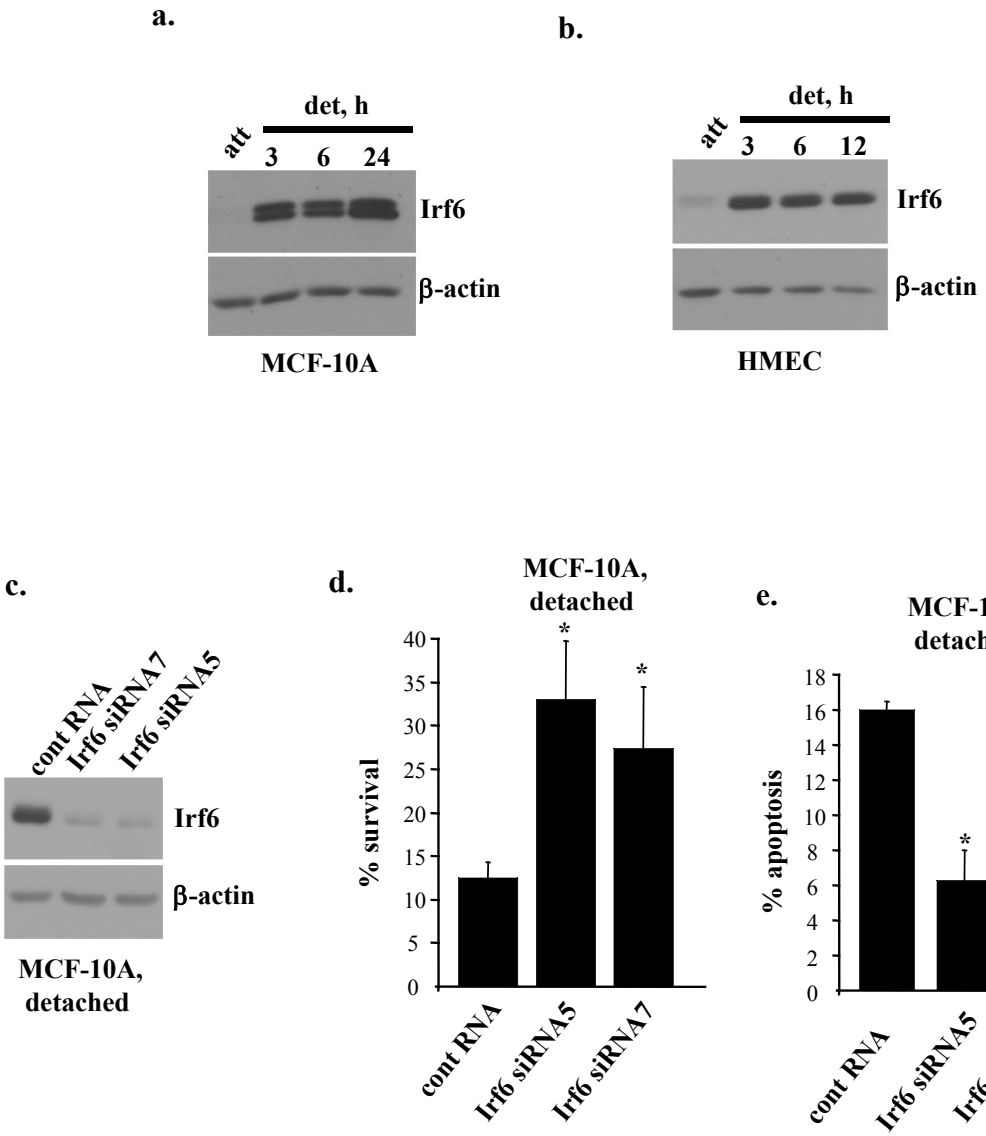

Figure 20. Detachment-induced upregulation of Irf6 is required for anoikis of non-malignant breast epithelial cells. (a, b) MCF10A cells (a) or human mammary epithelial cells (HMEC) (b) were cultured attached to (att) or detached from (det) the ECM for the indicated times and assayed for Irf6 levels by western blot. (c) MCF10A cells transfected with 100nM control RNA (cRNA) or Irf6-specific siRNA (Irf6siRNA) 5 or 7 were detached for 3h and assayed for Irf6 expression by western blot. β-actin was used as a loading control in a-c. (d) MCF10A cells treated as in (c) were allowed to form colonies in monolayer immediately or after 72h of detachment. % survival is % of colony number formed by the cells plated in monolayer immediately after transfection. The data represent the average of three independent experiments plus SE. (e) MCF10A cells treated as in (c) were detached for 48h, cell nuclei were stained with Hoechst 33258 and percentage of cells with condensed and/or fragmented nuclei (characteristic features of

apoptosis) in the total cell population (% apoptosis) was determined. The data represent the average of two independent experiments plus the SD. * - p-value is less than 0.05.

3.2.3 ErbB2-induced Irf6 downregulation is required for anoikis resistance of breast epithelial cells.

To test whether ErbB2-dependent Irf6 downregulation blocks anoikis of breast epithelial cells we reversed the effect of ErbB2 on Irf6. To this end, we transiently expressed ectopic HA-tagged-Irf6 in MCF-ErbB2 cells together with a Green Fluorescent Protein to visualize the transfected cells by fluorescence microscopy (Figure 21a). We found that exogenous Irf6 noticeably increased apoptosis of MCF-ErbB2 cells detached from the ECM (Figure 21b).

To examine the role of Irf6 in the regulation of anoikis of ErbB2-producing cells by a complementary technique we infected MCF-ErbB2 cells with a retrovirus encoding HA-tagged Irf6 (Figure 21c). We observed that exogenous Irf6 significantly increased apoptosis of detached MCF-ErbB2 cells (Figure 21d) and noticeably reduced their ability to form colonies without adhesion to the ECM in soft agar compared to the control cells (the latter ability is a well-known consequence of cancer cell anoikis resistance (Rosen et al., 1998; K. Rosen et al., 2000) (Figure 21e). Hence, ErbB2-induced Irf6 downregulation is required for anoikis resistance of ErbB2-overproducing breast epithelial cells.

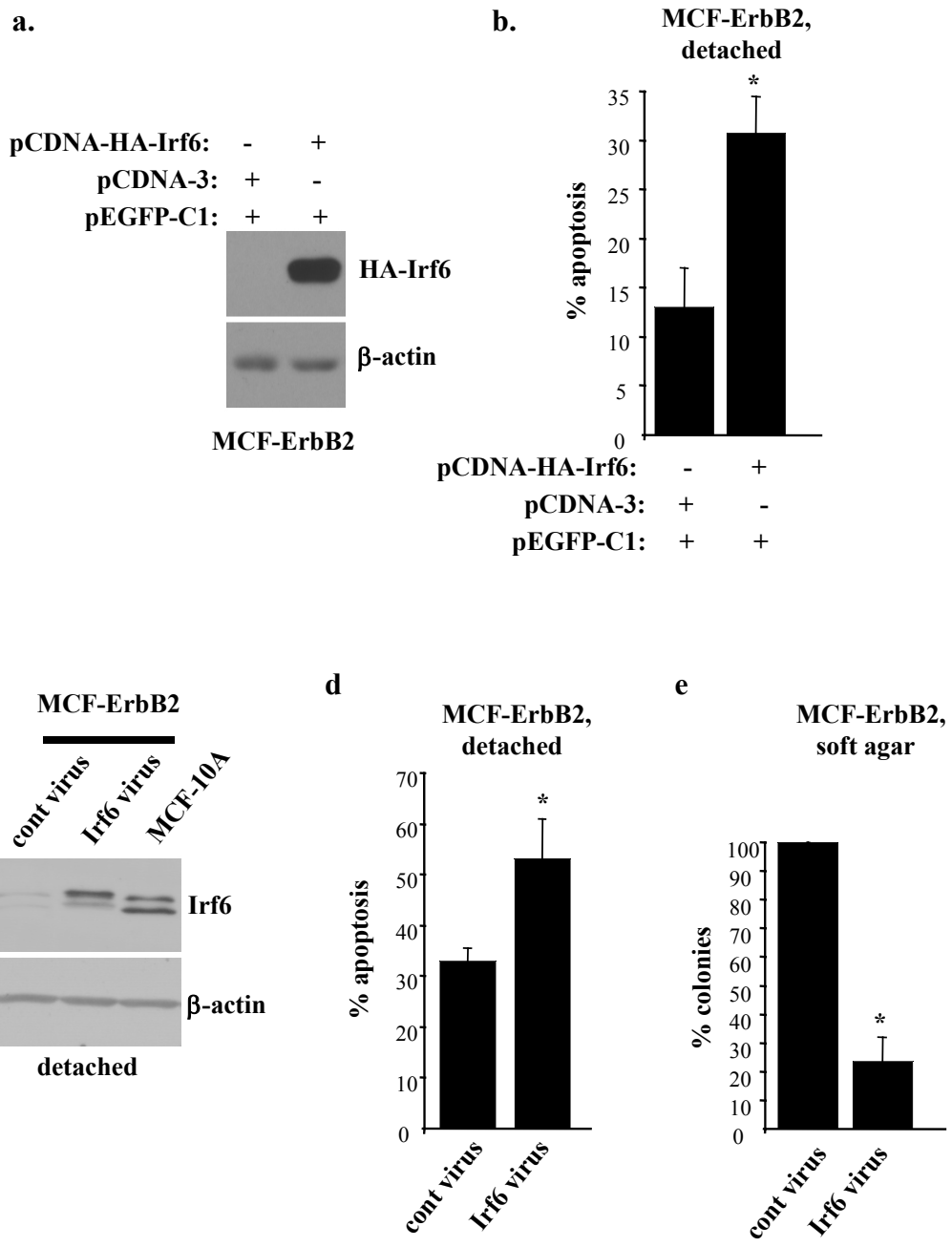

Figure 21. Downregulation of Irf6 is required for ErbB2-induced anoikis resistance of breast epithelial cells. (a) MCF-ErbB2 cells were transiently transfected (+) or not (-) with a control vector (pcDNA3) or pcDNA3 vector encoding HA-tagged Irf6 (pcDNA-HA-Irf6) and a vector encoding Green Fluorescent protein (GFP)(pEGFP-C1) and assayed for HA-Irf6 expression by western blot by use of anti-HA antibody. (b) Cells treated as in (a) were cultured detached from the ECM for 72h, cell nuclei were stained

with Hoechst 33258 and % of GFP-positive cells with condensed and/or fragmented nuclei (characteristic features of apoptosis) was determined as percentage of the cells with such nuclei in a population of GFP-positive cells. (c) MCF-ErbB2 cells infected with the control (cont virus) or the Irf6-encoding Maloney murine leukemia (MMLV) virus (Irf6 virus) were cultured detached from the ECM for 3h along with MCF10A cells and assayed for Irf6 levels by western blot. (d) MCF-ErbB2 cells treated as in (c) were detached from the ECM for 24h, stained with propidium iodide (PI) and assayed for Annexin V binding by flow cytometry. % apoptosis is the sum of % of Annexin V-positive/PI-negative cells and that of Annexin V-positive/PI-positive cells. (e) MCF-ErbB2 cells treated as in (c) were allowed to form colonies in soft agar. Number of colonies formed by the control cells was designated as 100%. The data in (b, d) are the average of the duplicates and those in (e) are the average of the triplicates plus the SD. β-actin was used as a loading control in (a, c). * - p-value is less than 0.05.

3.2.4 Detachment-induced Irf6 upregulation in non-malignant breast epithelial cells requires the presence of ΔNp63α.

One protein that can promote Irf6 transcription by binding the Irf6 promoter is ΔNp63, a member of the p53 family of transcription factors (Melino, 2011; Moretti et al., 2010). The p63 gene is transcribed from two different promoters to give rise to TAp63 or ΔNp63 that contain DNA-binding and oligomerization domains (Melino, 2011). In addition, TAp63 has an N-terminal transactivation (TA) domain. Both TAp63 and ΔNp63 exist as α-, β- or γ-isoforms generated via alternative splicing (Melino, 2011).

TAp63 and ΔNp63 both promote gene transcription, have numerous common and distinct targets (Trink, Osada, Ratovitski, & Sidransky, 2007). Importantly, ErbB2 causes loss of all p63 isoforms in a mouse model of breast cancer (Memmi et al., 2015), and none of the p63 isoforms are typically expressed in human breast tumours (Como et al., 2002). ΔNp63 can trigger both pro- and anti-apoptotic signals (DeYoung et al., 2006; Ihrie et al., 2005). Enforced downregulation of ΔNp63α in MCF10A cells was found to

trigger their epithelial-to-mesenchymal transition (J. Lindsay, McDade, Pickard, McCloskey, & McCance, 2011) while ErbB2-or Ras oncoprotein-induced ΔNp63α downregulation in MCF10A cells and other epithelial cell lines was shown to reduce migration and metastatic potential of respective cells (J. Lindsay et al., 2011).

We investigated the status of ΔNp63 in MCF10A cells by using ΔNp63-specific antibody validated for the detection of ΔNp63 isoforms by others (Curtis, Aenlle, Frisch, & Howard, 2015). Similar to published observations (J. Lindsay et al., 2011), we found that attached MCF10A cells produce only one ΔNp63 isoform, ΔNp63α (Figure 22a). The antibody validated by others for the detection of TAp63 (Curtis et al., 2015) did not react with any p63 species on respective western blots (not shown). We further noticed that when MCF10A cells detach from the ECM, ΔNp63α levels remain initially unchanged (for at least 6h) but ultimately decline (at 24h of suspension culture) (Figure 22a). Given that Irf6 is upregulated at least as early as at 3h of detachment (Figure 20a), we reasoned that ΔNp63α could mediate detachment-induced Irf6 upregulation while ΔNp63α levels in the detached cells are still high. Indeed, we observed that knockdown of p63 by two different siRNAs (Figure 22b) caused noticeable Irf6 downregulation in detached MCF10A cells (Figure 22c). Noteworthy, even though ΔNp63α levels are relatively high in the attached MCF10A cells (Figure 22a), this by itself is not sufficient for Irf6 upregulation (Figure 20a). Therefore, Irf6 is likely upregulated in the indicated cells by yet unidentified detachment-induced signals which are capable of upregulating Irf6 only in the presence of ΔNp63α.

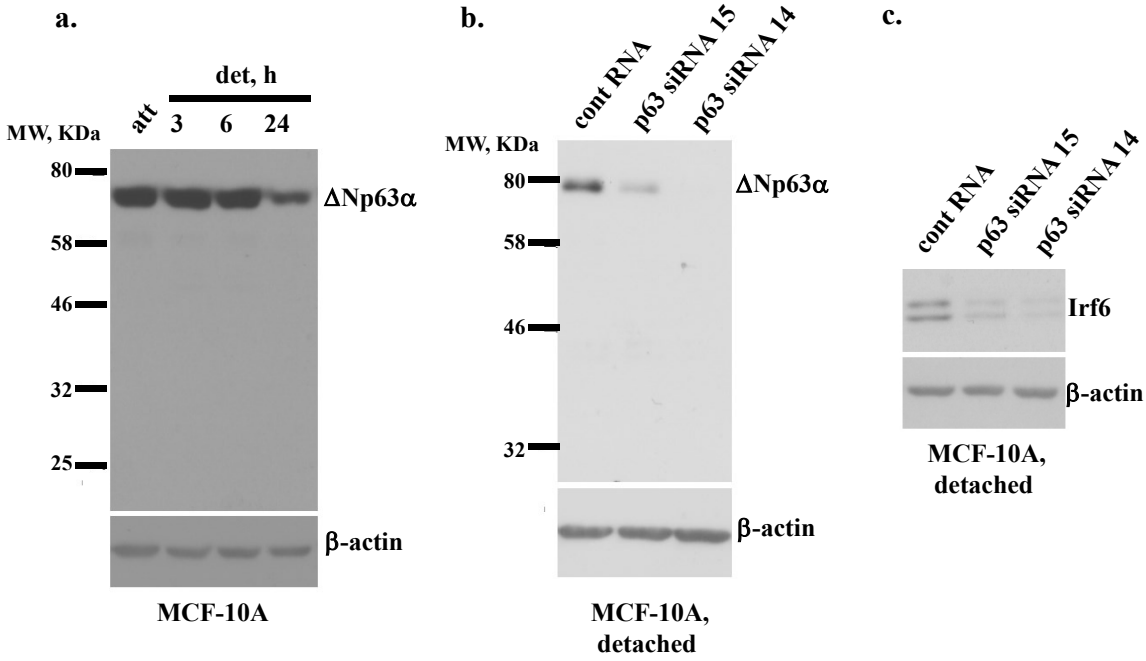

Figure 22. Detachment-induced upregulation of Irf6 in non-malignant breast epithelial cells requires the presence of ΔNp63α. (a) MCF10A cells were cultured attached to (att) or detached from (det) the ECM for the indicated times and assayed for ΔNp63α levels by western blot. (b, c) MCF10A cells transfected with 100nM control RNA (cRNA) or p63-specific siRNA (p63siRNA) 14 or 15 were detached for 24h and assayed for ΔNp63α (b) or Irf6 (c) expression by western blot. β-actin was used as a loading control.

3.2.5 ErbB2-induced downregulation of Irf6 in breast epithelial cells is mitogen-activated protein kinases-dependent.

Mitogen-activated protein kinases (MAPK) Erk1 and Erk2 are major mediators of ErbB2 signalling (Yarden & Sliwkowski, 2001). Thus, we further investigated whether the MAPK signalling pathway mediates the effect of ErbB2 on Irf6. We found that treatment with an Erk inhibitor SCH772984 (Hayes et al., 2016) significantly reduced phosphorylation of an Erk substrate Rsk (B. Yoo et al., 2017) and noticeably upregulated Irf6 in detached MCF-ErbB2 cells (Figure 23a). These data indicate that ErbB2 downregulates Irf6 in detached breast epithelial cells in an Erk-dependent manner.

We further found that a published derivative of MCF10A cells MCF-MekDD that we generated (Iman A. Khan, Yoo, Rak, & Rosen, 2014) by infection of MCF10A cells with a retrovirus encoding an activated mutant of Mek (an activator of Erk) displays significantly lower Irf6 levels than the parental MCF10A cells (Figure 23b). Thus, Mek activation is sufficient for Irf6 downregulation in detached breast epithelial cells.

Collectively, our data indicate that ErbB2 downregulates Irf6 in breast epithelial cells in a MAPK-dependent manner.

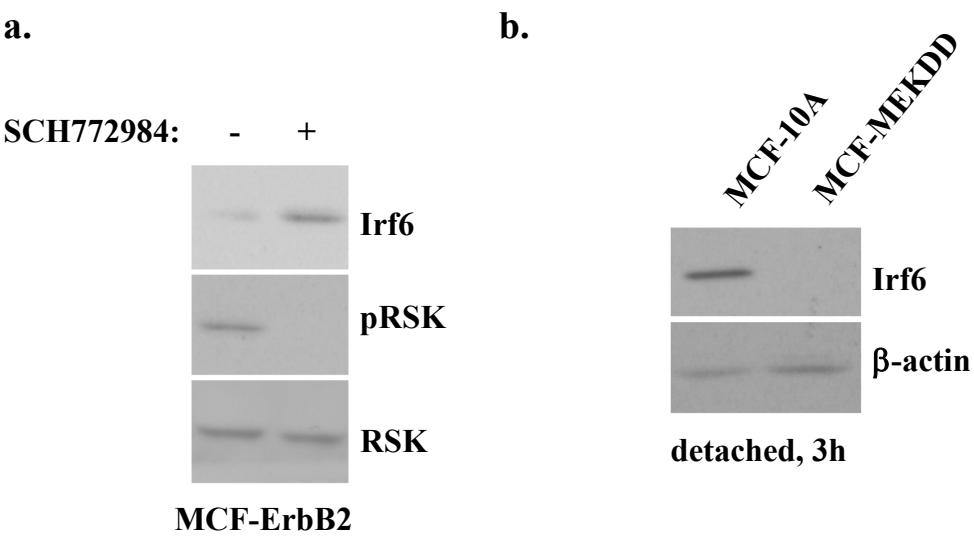

Figure 23.ErbB2-induced Irf6 downregulation occurs in a MAPK-dependent manner. (a) MCF-ErbB2 cells were cultured for 5h detached from the ECM in the presence of DMSO (-) or 1 μM SCH772984 (+) and assayed for Irf6 expression. The membrane was re-probed with an anti-phospho-RSK (pRsk) and then, an anti-RSK antibody. (b) MCF10A cells and a variant of MCF10A cells obtained by infection of these cells with a retrovirus carrying constitutively active Mek2 mutant Mek (MCF-MekDD) were cultured detached from the ECM for 3h and assayed for Irf6 expression by western blot. β-actin was used as a loading control.

3.2.6 ErbB2 inhibits signals that promote ΔNp63α-dependent-Irf6 upregulation in detached breast epithelial cells.

Since we found that ΔNp63α is required for detachment-induced Irf6 upregulation in non-malignant breast epithelial cells (Figure 22) we investigated the role of ΔNp63α in the effect of ErbB2 on Irf6. We noticed that both ErbB2 (Figure 24a-c) and an activated Mek mutant (Figure 24d-f) downregulate ΔNp63α in MCF10A cells.

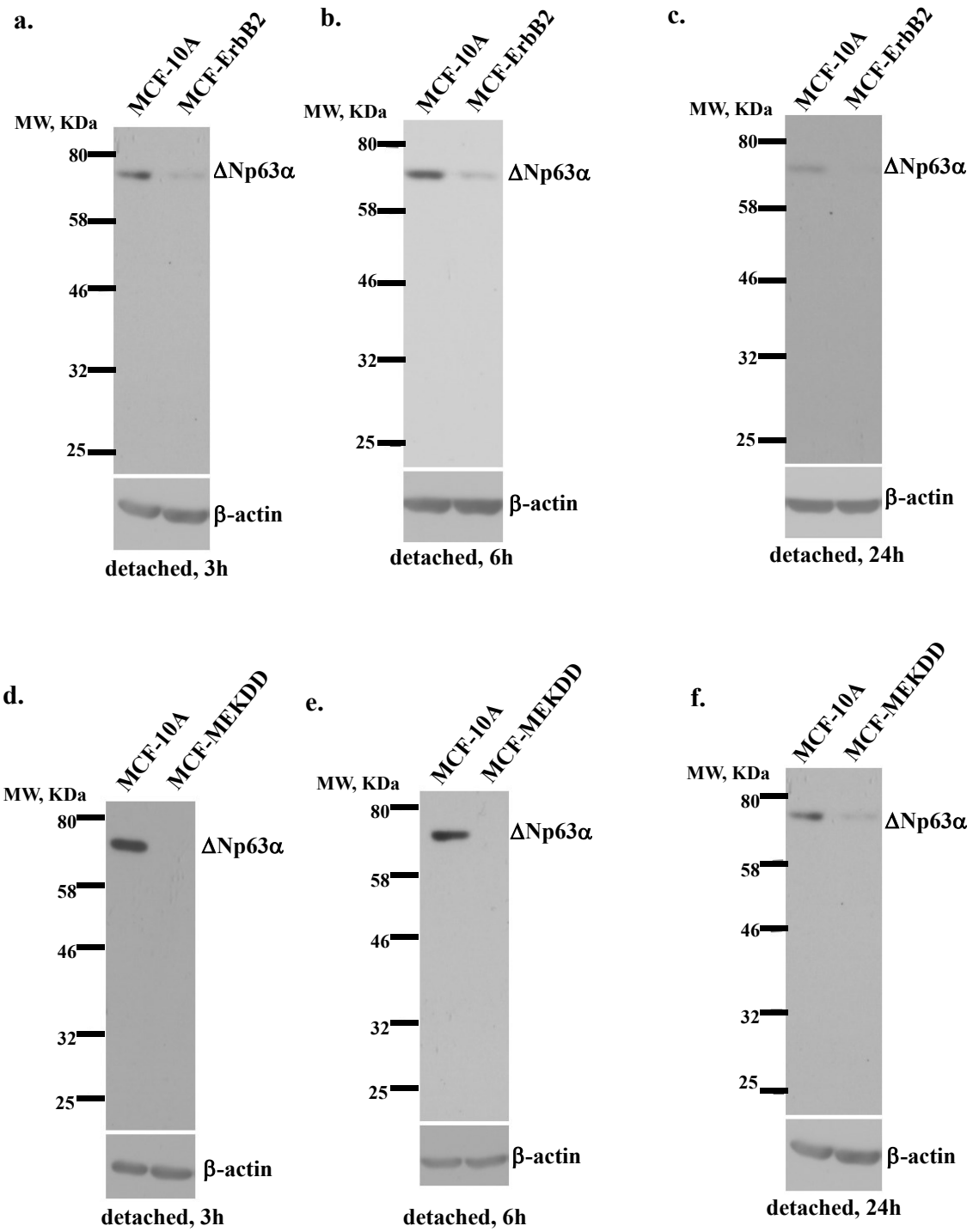
a.
MCF-10A
MCF-ErbB2
MW, KDa
80
58
46
32
25
ΔNp63α
β-actin
detached, 3h
b.
MCF-10A
MCF-ErbB2
MW, KDa
80
58
46
32
25
ΔNp63α
β-actin
detached, 6h
c.
MCF-10A
MCF-ErbB2
MW, KDa
80
58
46
32
25
ΔNp63α
β-actin
detached, 24h
d.
MCF-10A
MCF-MEKDD
MW, KDa
80
58
46
32
25
ΔNp63α
β-actin
detached, 3h
e.
MCF-10A
MCF-MEKDD
MW, KDa
80
58
46
32
25
ΔNp63α
β-actin
detached, 6h
f.
MCF-10A
MCF-MEKDD
MW, KDa
80
58
46
32
25
ΔNp63α
β-actin
detached, 24h

Figure 24. ErbB2 and Mek downregulate ΔNp63α in detached breast epithelial cells. MCF10A and MCF-ErbB2 cells (a-c) or MCF10A and MCF-MekDD cells (d-f) were cultured detached from the ECM for the indicated times and assayed for ΔNp63α levels by western blot. β-actin was used as a loading control.

We further observed that an Erk inhibitor SCH772984 (Hayes et al., 2016) upregulates ΔNp63α in detached MCF10-ErbB2 cells (Figure 25a). Moreover, we found that when this upregulation is blocked by two different ΔNp63α-specific siRNAs (Figure 25a), the Erk inhibitor fails to upregulate Irf6 in detached MCF-ErbB2 cells (Figure 25b). As expected, the inhibitor efficiently blocked phosphorylation of the Erk substrate Rsk (B. Yoo et al., 2017) in all cases (Figure 25c). These data indicate that ErbB2/MAPK downregulates Irf6 by blocking ΔNp63α-dependent signalling mechanisms in detached cells. Noteworthy, in case of non-malignant breast epithelial cells detachment-induced signalling events can upregulate Irf6 only in the presence of ΔNp63α (Figures 22 and 25e). Hence, ErbB2/MAPK signalling could downregulate Irf6 in detached MCF-ErbB2 cells by blocking either the indicated signalling events or by downregulating ΔNp63α itself.

To distinguish between these possibilities, we tested whether the reversal of the effect of ErbB2 on ΔNp63α blocks the effect of ErbB2 on Irf6. To this end, we infected MCF-ErbB2 cells with a retrovirus encoding ΔNp63α (Figure 25d) and tested whether ectopic ΔNp63α upregulates Irf6 in the detached cells. We found that ectopic ΔNp63α does not upregulate Irf6 in these cells (Figure 25d). These data indicate that ErbB2/MAPK-driven signals block both detachment-induced signalling events that promote ΔNp63α-dependent Irf6 upregulation and, in addition, downregulate ΔNp63α itself in detached MCF-ErbB2 cells (see Figure 25 e, f for models describing these

scenarios). When Erk activity is blocked, both of these events are reversed and Irf6 is upregulated (Figure 25a, b). However, in the absence of the indicated detachment-induced signals ectopic ΔNp63α by itself is unable to upregulate Irf6.

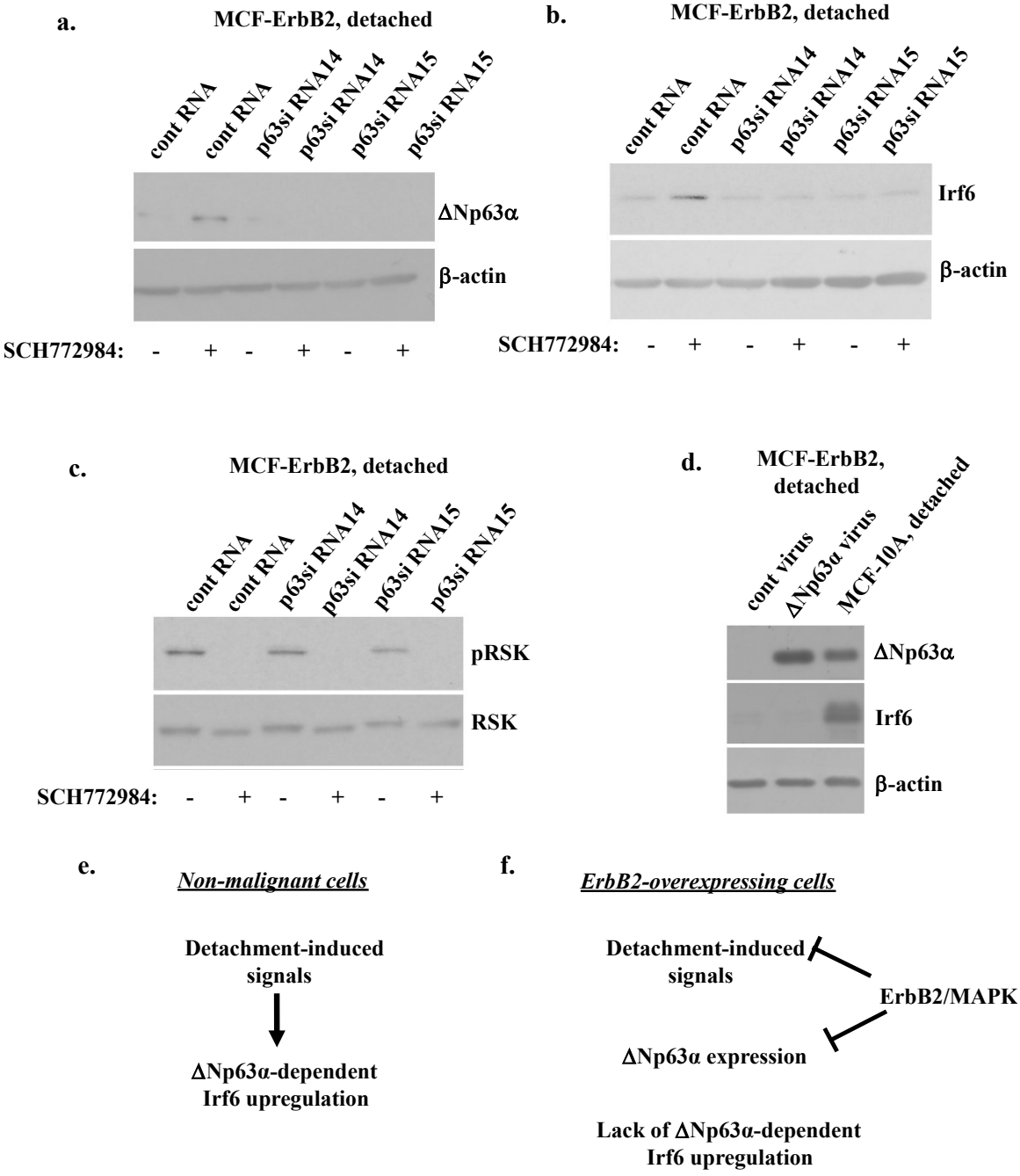

Figure 25. Erk blocks ΔNp63α-dependent signals that upregulate Irf6 in detached ErbB2-overproducing breast epithelial cells. (a-c) MCF10A cells transfected with 100nM control RNA (cRNA) or p63-specific siRNA (p63siRNA) 14 or 15 were detached for 24h in the presence of DMSO (-) or 1 μM SCH772984 (+) and assayed for ΔNp63α (a), Irf6 (b) or phospho-Rsk (pRsk) and Rsk (c) expression by western blot. (d) MCF-

ErbB2 cells were infected with the control (cont virus) or the ΔNp63α-encoding MMLV, cultured detached from the ECM for 3h along with MCF10A cells and assayed for ΔNp63α levels by western blot. β-actin was used as a loading control. (e, f) Schematic representation of a model of events that take place in detached non-malignant (e) and ErbB2-overproducing (f) breast epithelial cells. (e) Detachment-induced signals can upregulate Irf6 in the non-malignant cells only in the presence of ΔNp63α (f) ErbB2 blocks both the indicated detachment-induced signals and ΔNp63α expression in detached breast cancer cells. In the absence of detachment-induced signals Irf6 is not upregulated in Erbb2-overproducing cells.

3.2.7 Irf6 regulates Perp expression in breast epithelial cells.

We further investigated the molecular mechanism by which Irf6 promotes anoikis of breast epithelial cells. Irf6 DNA binding domain has been shown to bind to a consensus DNA binding sequence 5'- A (C/T) (C/T) (G/C) A (A/T) AC -3' (Botti et al., 2011; Little et al., 2008). Transcription factors commonly bind to such sites or those complementary to them (Lang & Spandidos, 1986). With the help of a software called PROMO (a bioinformatics tool) provided by ALGGEN (Algorithmic and Genetics) group from Polytechnic University of Catalonia, Barcelona, Spain, we have found putative Irf6 binding sites on the Perp gene across various species between positions -950 and -750 (Figure 26). These sites are complementary to the previously published Irf6 consensus binding site (Botti et al., 2011).

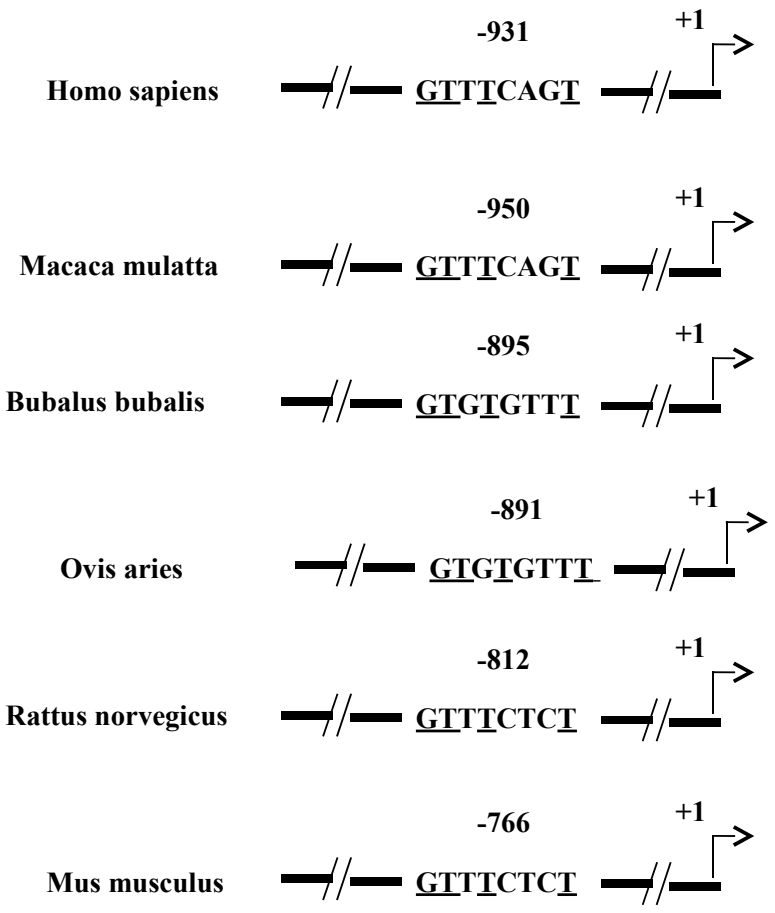

Figure 26. Schematic representation of the location of the potential Irf6 binding sites in the DNA regions preceding transcription start site of the Perp gene in various species. The sequence of the consensus Irf6 binding site is A(C/T)(C/T)(G/C)A(A/T)AC (any of the nucleotides in parentheses could be present at a given position of this sequence)(Botti et al., 2011). The sequence complementary to this consensus site is GT(T/A/G) T(C/G) (G/A) (G/A) T. Positions of the DNA sequences that are closely related to the latter sequence are shown for the human (homo sapiens), rhesus macaque (macaca mulatta), water buffalo (bubalus bubalis), sheep (ovis aries), rat (rattus norvegicus) and mouse (mus musculis) Perp genes. Nucleotides in the sequences shown in the figure that correspond to the invariable nucleotides in Irf6 consensus binding site (Botti et al., 2011) GT(T/A/G)T(C/G)(G/A)(G/A)T are underlined.

In order to test whether Irf6 regulates Perp expression in detached MCF10A cells we ablated Irf6 by using two Irf6-specific siRNAs and observed a noticeable reduction in Perp expression compared to cells transfected with control RNA (Figure 27a). Furthermore, we observed that exogenous expression of Irf6 at physiological levels in attached MCF10A cells achieved by infecting the cells with retroviruses encoding either full-length Irf6 or empty vector (Figure 27b) upregulated Perp expression (Figure 27c) in these cells (which otherwise is known to be reduced in attached breast epithelial cells (Figure 12a) (I. A. Khan et al., 2016)). Therefore, our evidences suggest that Irf6 regulates Perp expression in breast epithelial cells and perhaps may contribute to Perp-mediated apoptosis of breast epithelial cells.

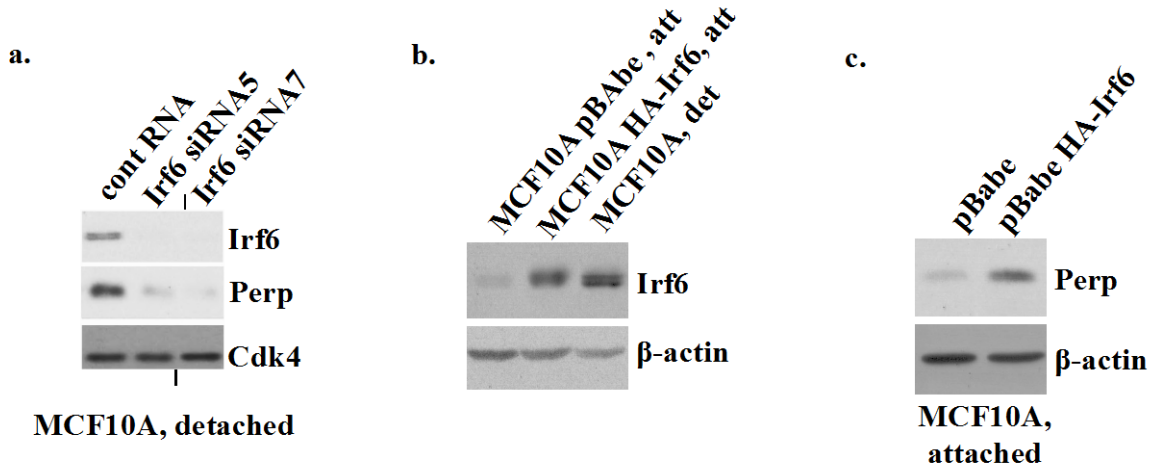

Figure 27. Irf6 regulates Perp expression in breast epithelial cells. (a) MCF10A cells treated as in Figure 20c were assayed for Perp expression by western blot. Cdk4 served as a loading control. (b) MCF10A cells infected with the control (MCF10A pBabe) virus or the Irf6-encoding Maloney murine leukemia virus (MCF-ErbB2 HA-Irf6) were cultured attached on the ECM for 48 h and assayed for Irf6 levels by western blot. MCF10A cells cultured detached from the ECM for 3 h were used as positive control. (c) MCF10A derivatives (pBabe) and (HA-Irf6) were treated as in (d) and assayed for Perp expression

by western blot. β-actin served as loading control for (b) and (c). When lanes were removed from western blot images, and separate parts of an image were joined together, a short vertical black line was used to indicate where the image was cut.

3.2.8 Summary

In summary, we have identified a novel mechanism by which ErbB2 promotes anoikis resistance of breast cancer cells. This mechanism is driven by ErbB2-induced downregulation of transcription factor Irf6 in detached breast epithelial cells requires concomitant downregulation of a transcription factor ΔNp63α. Furthermore, Irf6-induced anoikis of breast epithelial cells is suggested to be mediated by a known anoikis inducer Perp (I. A. Khan et al., 2016).

3.3 Mek activity is required for ErbB2 expression in breast cancer cells detached from the extracellular matrix.

Further investigation of the mechanisms involved in ErbB2-induced anoikis resistance of breast cancer cells revealed that ErbB2 expression in detached breast cancer cells requires activation of Mek kinase. The text of this chapter is presented verbatim as it was published by us (Iman A. Khan et al., 2014) with the exception of those cases when the text was modified according to the Dalhousie University Thesis guidelines. See Appendix A for author contributions and Appendix C for the permission from Oncotarget.

3.3.1 Mek activity is required for ErbB2 expression in detached breast cancer cells.

We found that treatment of MCF10A cells with a widely used highly specific Mek inhibitor selumetinib (Akinleye, Furqan, Mukhi, Ravella, & Liu, 2013; Corcoran et al., 2013) strongly downregulates ErbB2 in detached MCF- ErbB2 cells but has no impact on ErbB2 in the attached cells (Figure 28a). The effect of selumetinib on ErbB2 was not unique to MCF-ErbB2 cells as we found that the Mek inhibitor downregulates ErbB2 in detached ErbB2- positive human breast cancer cell lines BT-474, AU-565 and HCC-1419 (N. A. O'Brien et al., 2010; Subik et al., 2010) but has no effect on ErbB2 levels when these cells are attached to the ECM (Figure 28b-d). Thus, Mek activity is required for ErbB2 expression in breast cancer cells detached from the ECM.

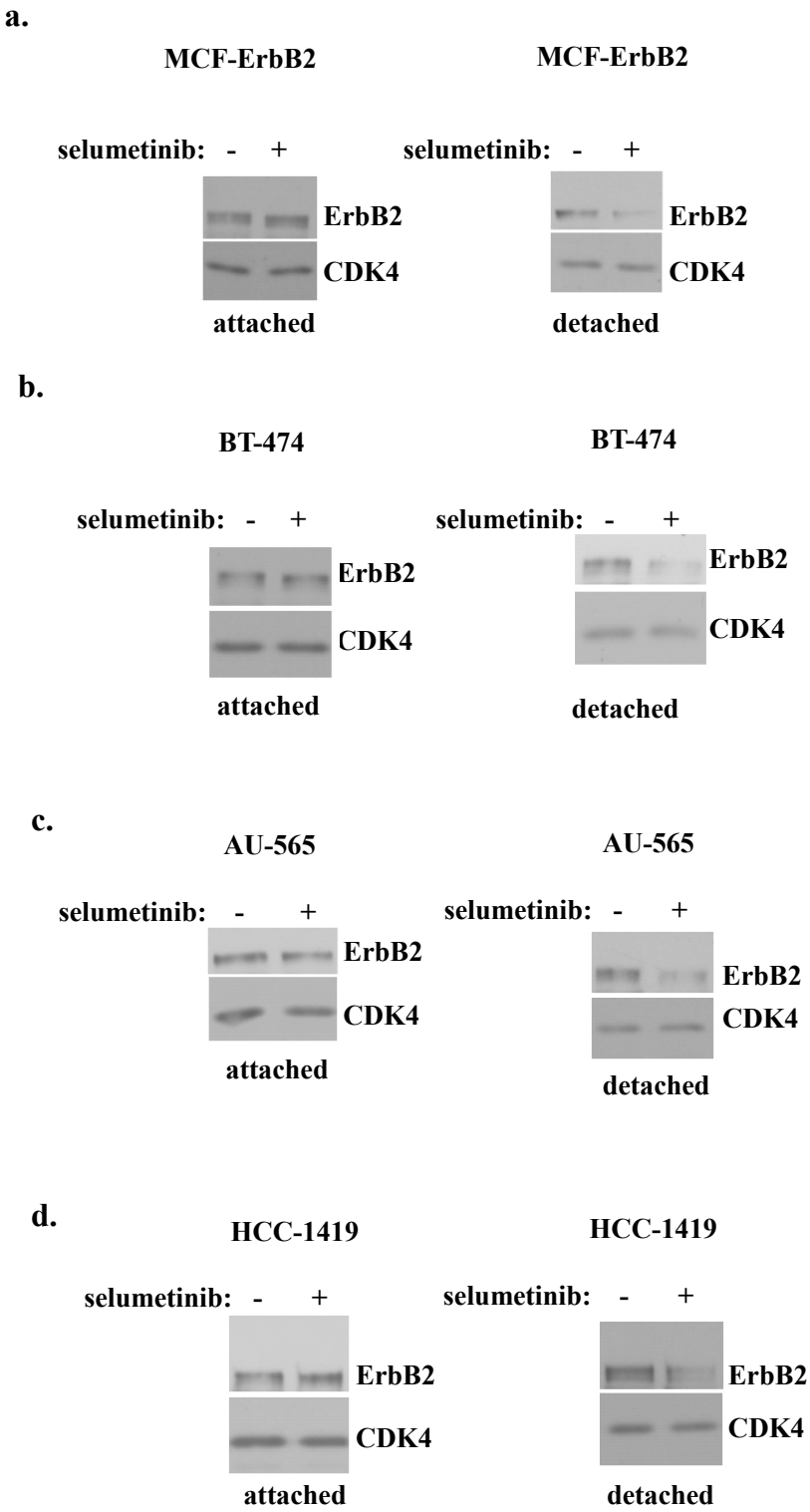
a.
MCF-ErbB2
selumetinib: - +
ErbB2
CDK4
attached
MCF-ErbB2
selumetinib: - +
ErbB2
CDK4
detached
b.
BT-474
selumetinib: - +
ErbB2
CDK4
attached
BT-474
selumetinib: - +
ErbB2
CDK4
detached
c.
AU-565
selumetinib: - +
ErbB2
CDK4
attached
AU-565
selumetinib: - +
ErbB2
CDK4
detached
d.
HCC-1419
selumetinib: - +
ErbB2
CDK4
attached
HCC-1419
selumetinib: - +
ErbB2
CDK4
detached

Figure 28. Mek activity is required for ErbB2 expression in breast cancer cells detached from the ECM. MCF-ErbB2 (a), BT-474 (b), AU-565 (c) and HCC-1419 cells (d) were cultured attached to (attached) or detached from (detached) the ECM in the presence of DMSO (-) or 1μM selumetinib (+) for 5h and assayed for ErbB2 expression by western blot. CDK4 was used as a loading control.

3.3.2 Mek-dependent ErbB2 expression is required for anchorage-independent growth of malignant breast epithelial cells.

We further tested whether selumetinib blocks the ability of ErbB2-overproducing breast epithelial cells MCF-ErbB2 to grow without adhesion to the ECM. Of note, Mek inhibition is known to trigger various feedback anti-apoptotic mechanisms in cancer cells (e.g. ErbB3 upregulation (C. Sun et al., 2014) which could in principle promote their survival. Thus, the effect of Mek inhibition on anchorage- independent growth of ErbB2-positive cells cannot be predicted. We observed that selumetinib-induced ErbB2 downregulation in MCF-ErbB2 cells (see Figure 28a) is accompanied by loss of the ability of these cells to grow anchorage-independently as colonies in soft agar (Figure 29a). Moreover, we found that ErbB2 knockdown in MCF-ErbB2 cells by small interfering (si) RNAs can mimic the effect of selumetinib on these cells, i.e. such knockdown strongly blocks their soft agar growth (Figure 29b-c).

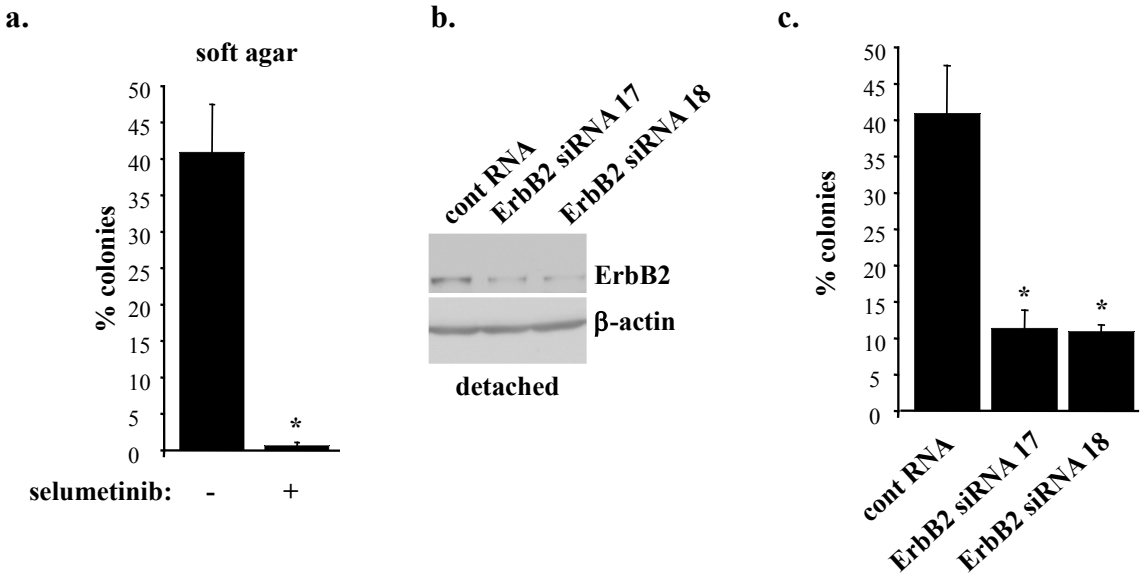

Figure 29. Inhibition of Mek or ErbB2 knockdown block anchorage-independent growth of ErbB2-overproducing breast epithelial cells. (a). MCF-ErbB2 cells were plated in monolayer culture or in soft agar in the presence of DMSO (-) or 1μM selumetinib (+) and allowed to form colonies which were counted 10 days later. % colonies were calculated as a ratio between the number of colonies in soft agar to that in monolayer x100%. (b). MCF-ErbB2 cells were transfected with 25nM control RNA or 25 nM ErbB2-specific siRNA (ErbB siRNA) 17 or 18, cultured detached from the ECM for 24 and assayed for ErbB2 expression by western blot. β-actin was used as a loading control. (c). Cells treated as in (b) were assayed as in (a). The data in (a) and (c) represent the average of the triplicates plus SD. * indicates that p value was < 0.05.

We further tested the effect of Mek on ErbB2 in detached MCF10A cells and a variant of these cells MCF-MekDD generated by infection of the former cells with the retrovirus encoding an activated Mek mutant (Haenssen et al., 2010; I. A. Khan et al., 2016). We found that as expected MCF-MekDD cells displayed higher total Mek levels than the parental MCF10A cells (Figure 30a) and showed much higher ErbB2 levels than MCF10A cells when these cell lines were detached from the ECM (Figure 30b). We

further observed that MCF-MekDD cells are capable of growing anchorage-independently in soft agar and that treatment with trastuzumab, a therapeutic anti-ErbB2 antibody (Leyland-Jones, 2002), significantly reduced the ability of the indicated cells to grow in this manner (Figure 30c). Thus, one function of Mek in detached breast cancer cells is to support ErbB2 expression. In the absence of active ErbB2 the ability of Mek to promote anchorage- independent growth of such cells is significantly reduced.

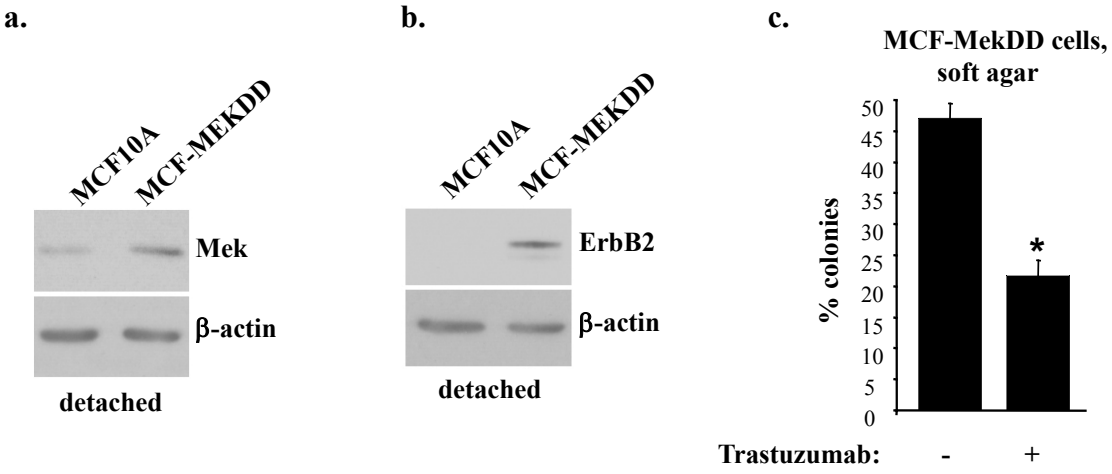

Figure 30. Mek-induced ErbB2 upregulation promotes anchorage-independent growth of breast epithelial cells. Indicated cells were cultured detached from the ECM for 3h and assayed for Mek (a) or ErbB2 (b) expression by western blot. β-actin was used as a loading control. (c). MCF-MekDD cells were plated in monolayer culture or in soft agar in the absence (-) or in the presence (+) or 5μg/ ml trastuzumab and allowed to form colonies which were counted 10 days later. % colonies were calculated as a ratio between the number of colonies in soft agar to that in monolayer x100%. The data represent the average of the triplicates plus SD. * indicates that p value was < 0.05.

3.3.3 Mek does not upregulate ErbB2 mRNA in detached breast cancer cells.

In an effort to understand the mechanisms by which Mek controls ErbB2 expression in detached breast cancer cells we found that selumetinib does not downregulate ErbB2 mRNA in detached MCF-ErbB2 cells. To the contrary, the Mek inhibitor upregulated this mRNA in the indicated cells (Figure 31a). We also observed that selumetinib has no effect on the ErbB2 mRNA in detached human breast cancer cells BT474 (Figure 31b). Moreover, the Mek inhibitor upregulated, rather than downregulated, the ErbB2 mRNA in detached human breast cancer cells HCC-1419 (Figure 31c). Finally, an activated Mek mutant, which upregulates ErbB2 protein in detached MCF-10A cells (Figure 31b), did not upregulate the ErbB2 mRNA in these cells (Figure 31d). Hence, since selumetinib downregulates ErbB2 protein in detached breast cancer cells (Figure 28), whereas activated Mek upregulates ErbB2 protein in detached breast epithelial cells (Figure 30b), the effect of Mek on ErbB2 protein in such cells does not involve changes in the ErbB2 mRNA.

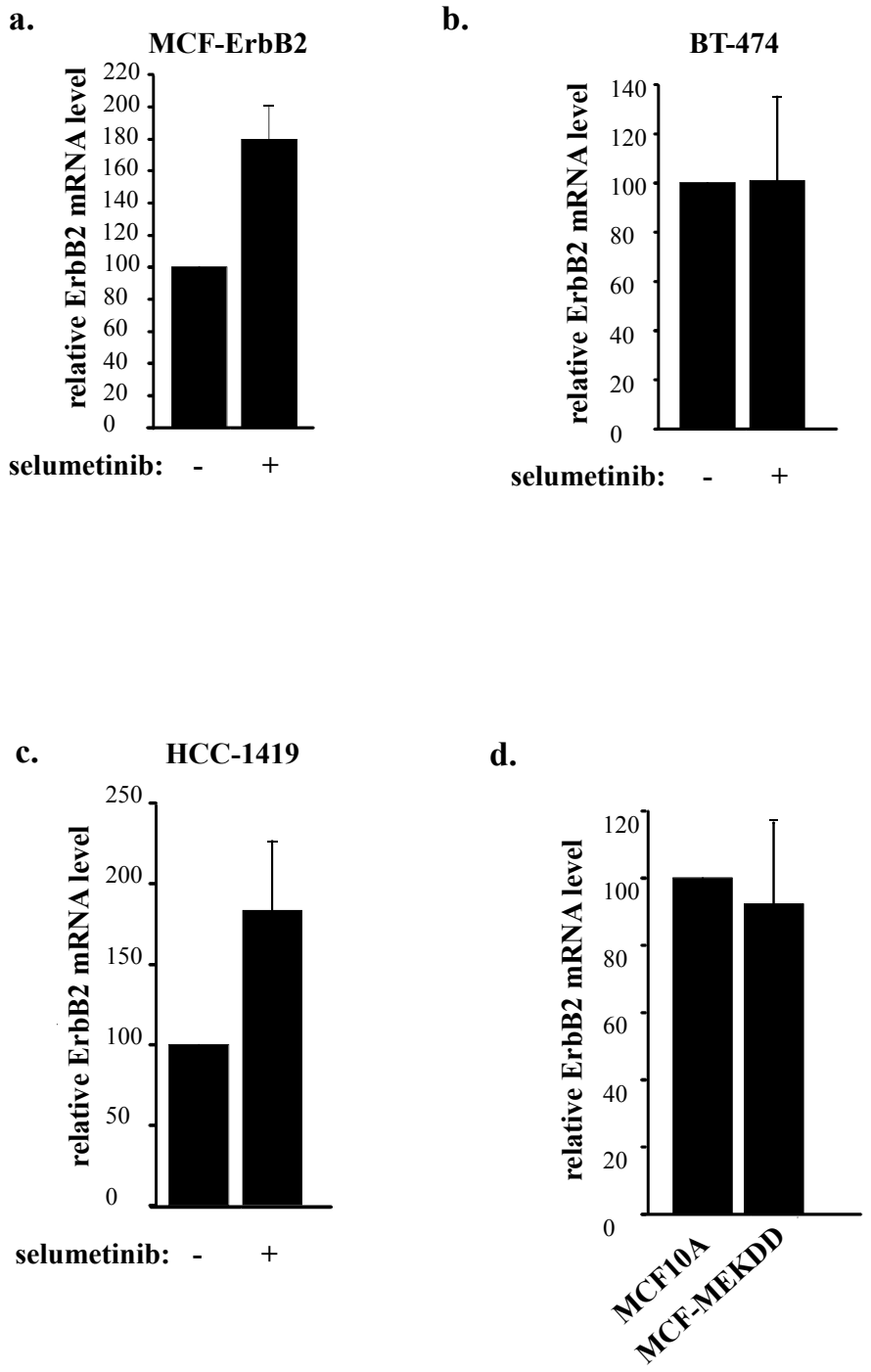

Figure 31. Mek does not control ErbB2 mRNA expression in breast cancer cells detached from the ECM. MCF-ErbB2 (a), BT-474 (b) and HCC-1419 (c) were cultured detached from the ECM (detached) in the presence of DMSO (-) or 1μM selumetinib (+) for 5h and assayed for ErbB2 mRNA levels by qPCR. (d). MCF10A and MCF-MekDD

cells were cultured detached from the ECM for 3h and assayed for ErbB2 mRNA levels by qPCR. ErbB2 mRNA levels observed in (a-d) were normalized by the levels of 18S rRNA which were also determined by qPCR. The resulting levels of the ErbB2 mRNA in the DMSO-treated cells (a-c) or MCF10A cells (d) were designated as 100%. The data in (a, c) represent the average of three and those in (b, d) the average of four independent experiments plus SE.

3.3.4 ErbB2 loss following Mek inactivation in detached breast cancer cells can be blocked by a lysosomal inhibitor.

Given that Mek inhibition downregulates ErbB2 protein in detached breast cancer cells without downregulating the ErbB2 mRNA, we tested whether Mek inactivation blocks ErbB2 protein stability in these cells. To this end, we treated detached human breast cancer cells BT474 with a protein synthesis inhibitor cycloheximide (B. Yoo, Berezkin, Wang, Zagryazhskaya, & Rosen, 2012; B. H. Yoo et al., 2010). As could have been expected in the absence of *de novo* protein synthesis, ErbB2 levels were reduced to some extent by this treatment (Figure 32a). Moreover, cycloheximide noticeably enhanced selumetinib-induced ErbB2 protein loss in detached breast cancer cells (Figure 32a). These data are consistent with a scenario that the absence of *de novo* protein synthesis accelerates selumetinib-induced ErbB2 protein loss resulting from selumetinib-induced ErbB2 protein degradation.

We further tested whether Mek inhibition promotes covalent binding of ErbB2 to a protein ubiquitin and subsequent ErbB2 proteasomal degradation (a known mechanism of protein turnover (Haller et al., 2014) in detached breast cancer cells. To this end, we treated detached BT474 cells with selumetinib in the absence and in the presence of a proteasomal inhibitor MG132 (Haller et al., 2014). We observed that treatment with

MG132 for 2h (not shown) or 5h (Figure 32b) does not block the effect of selumetinib on ErbB2 in these cells. Hence, ErbB2 ubiquitination and subsequent proteasomal degradation does not seem to be involved in the effect of selumetinib on ErbB2 in detached breast cancer cells.

It is known that Mek inhibition accelerates lysosomal degradation of EGFR (Grassian, Schafer, et al., 2011), an ErbB2 signalling partner (Harari & Yarden, 2000). Moreover, lysosomal degradation is an established mechanism of ErbB2 protein turnover in certain circumstances. For example, it was found that a therapeutic HSP90 inhibitor (Castagnola et al., 2014) or an inhibitor of phosphatidylcholine-specific phospholipase C (Paris et al., 2010) trigger lysosomal ErbB2 degradation in breast cancer cells. To test whether Mek inhibition promotes degradation of ErbB2 in the lysosomes of detached breast cancer cells we treated detached BT474 cells with selumetinib in the absence and in the presence of a lysosomal inhibitor Bafilomycin A1 (B. Yoo et al., 2009; B. Yoo et al., 2015). We observed that Bafilomycin A1 strongly blocks selumetinib-induced ErbB2 downregulation in these cells (Figure 32c). Collectively, our data are consistent with a scenario that Mek activity prevents lysosomal ErbB2 degradation in detached breast cancer cells.

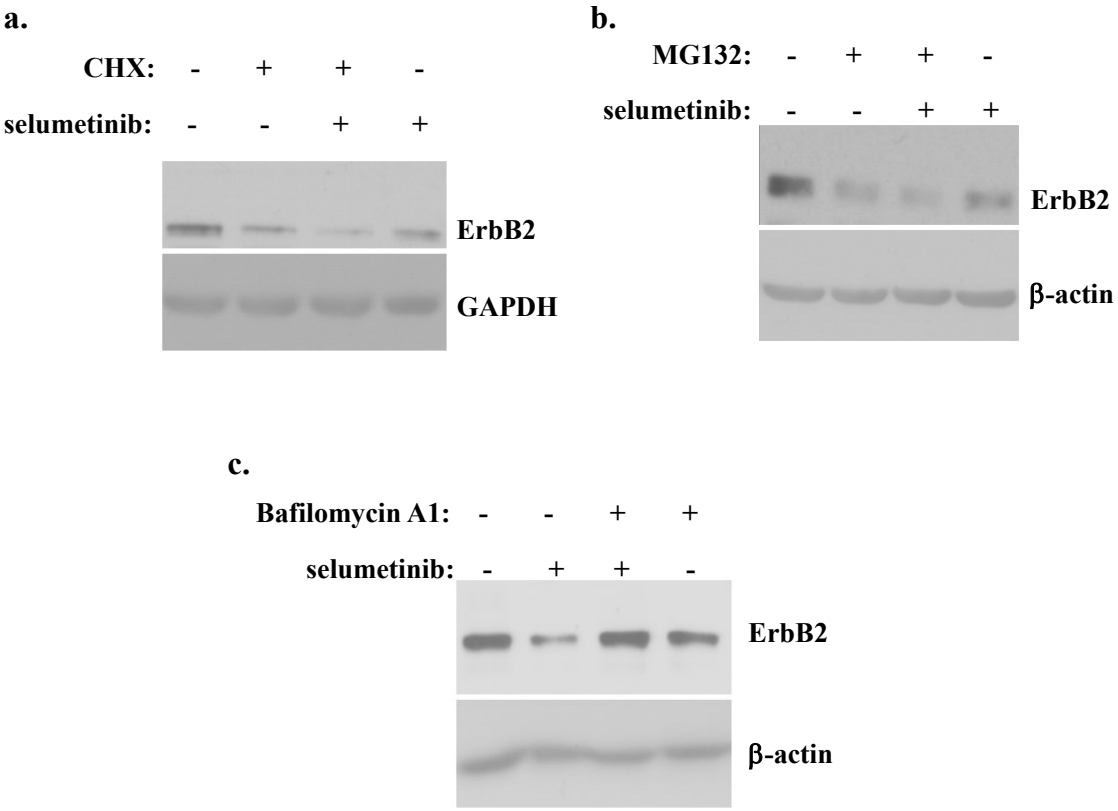

Figure 32. ErbB2 downregulation induced by a Mek inhibitor in detached breast cancer cells can be blocked by a lysosomal inhibitor. (a) BT-474 cells were cultured detached from the ECM in the presence of DMSO (-) or 1μM selumetinib (+) or 10μg/ml cycloheximide (CHX) (+) for 3h and assayed for ErbB2 expression by western blot. (b) BT-474 cells were cultured detached from the ECM in the presence of DMSO (-) or 1μM selumetinib (+) or 10μg/ml MG132 (MG132) (+) for 5h and assayed for ErbB2 expression by western blot. (c) BT-474 cells were cultured detached from the ECM in the presence of DMSO (-) or 1μM selumetinib (+) or 100 nM Bafilomycin A1 (+) for 5h and assayed for ErbB2 expression by western blot. GAPDH (a) and β-actin (b-c) was used as a loading control.

Of note, it was proposed that in certain circumstances ErbB2 can covalently bind ubiquitin, and that such ubiquitination further triggers proteasome- mediated cleavage of the intracellular ErbB2 region (Bertelsen & Stang, 2014). This, in turn was suggested to induce internalization and subsequent sorting of ErbB2 to the lysosomes (Bertelsen & Stang, 2014). Our data showing that selumetinib-induced ErbB2 degradation is proteasome-independent (Figure 32b) indicate that the mechanism outlined above is unlikely involved in the effect of selumetinib on ErbB2 in breast cancer cells detached from the ECM.

3.3.5 The activity of the Mek/Erk Signalling pathway, ErbB2 expression and trastuzumab sensitivity can be blocked by an increased density of detached breast tumour cells.

Our data indicate that Mek inhibition blocks ErbB2 expression in detached breast cancer cells. In search for physiologically relevant circumstances under which this scenario could take place we reasoned that ErbB2 loss represents an established mechanism of breast cancer resistance to treatment with a therapeutic anti-ErbB2 antibody trastuzumab (Burnett et al., 2015; Guarneri et al., 2013). Moreover, it was found that breast tumour cells circulating in patient's blood are ErbB2-positive in a significant number of cases whereas the cells composing respective primary tumours are often ErbB2-negative (Wülfing et al., 2006). Finally, it was found that growth of tumours formed by ErbB2-positive breast cancer cells injected in immunodeficient mice is blocked by trastuzumab significantly more efficiently if the drug is administered immediately after cell injection (before the tumour mass is formed) compared to the situation when the drug is administered after a tumour mass is established (Ithimakin et

al., 2013). Thus, we speculated that increase in the cancer cell density within a three-dimensional tumour mass can block the activity of Mek or that of its substrates and cause inhibition of ErbB2 expression. This in turn could render breast cancer cells trastuzumab-resistant.

To test whether an increased density of detached breast cancer cells can block the activity of Mek or that of its substrate Erk and inhibit ErbB2 expression we compared the levels of Erk1 and Erk2 (the two Mek substrates (Mandal, Becker, & Strebhardt, 2015), phospho-Erk1 and phospho-Erk2 as well as that of ErbB2 in human breast cancer cells BT-474 detached from the ECM grown as a "sparse" culture to those in detached BT-474 cells grown at a 10 times higher concentration (which we refer to as a "dense" culture). The cells in the sparse culture formed multiple relatively small size spheroids whereas those cultured in the dense culture represented one large multicellular aggregate (not shown). We found that densely grown cells display much lower levels of Erk1 and Erk2 proteins as well as those of phospho-Erk1 and phospho-Erk2 than the sparsely grown cells (Figure 33a). Of note, the increase in cell density did not significantly reduce the levels of the Erk1 and Erk2 mRNAs (data not shown) indicating that the observed downregulation of Erk1 and Erk2 occurred at the protein, rather than at the mRNA level. Unlike the case with Erk and phospho-Erk, the indicated increase in the cell density did not significantly alter the cellular protein levels of Mek and phospho-Mek (Figure 33b). We further found that an increase in the density of detached cells resulted in a dramatic reduction of ErbB2 protein levels (Figure 33a).

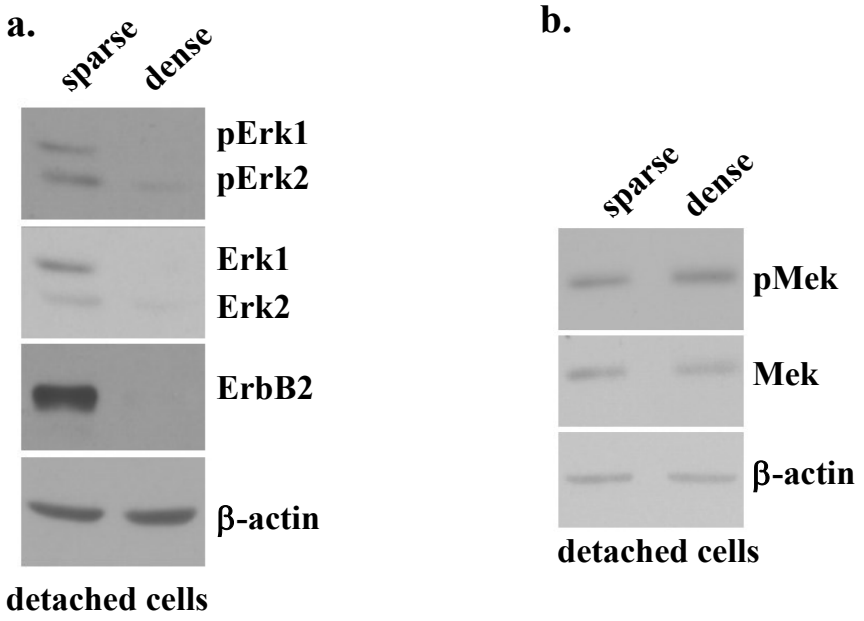

Figure 33. pErk, Erk and ErbB2 protein expression is blocked by an increased density of detached breast tumour cells. (a, B) BT-474 cells were cultured detached from the ECM for 6 days at a concentration 12500 cells/ml (sparse) or 125000 cells/ml (dense) and assayed for phospho-Erk1 (pErk1), phospho-Erk2 (pErk2), Erk1, Erk2 and ErbB2 protein expression (a) or phospho-Mek (pMek) and Mek protein expression (b) by western blot. β-actin was used as a loading control in (a-b).

We subsequently noticed that culturing detached cells sparsely did not reduce their number compared to the number of those initially plated (Figure 34a). In contrast, culturing the cells at high density significantly reduced their number compared to the number of initially plated cells (Figure 34b). These data are consistent with our observations shown in Figures 28-30 indicating that ErbB2 is required for anchorage-independent growth of these cells. Of note, even though plating the cells in the dense

culture noticeably reduced their number, a substantial fraction of the cells remained viable (Figure 34b). One possible explanation of the fact that these cells survived in the absence of ErbB2 is that in addition to ErbB2 gene amplification (N. A. O'Brien et al., 2010), other oncogenic changes present in the cells contribute to their ability to grow anchorage- independently. For example, BT474 cells also carry a loss-of-function mutation of the p53 tumour suppressor gene (Papanikolaou et al., 2009), and loss of p53, a well-known inhibitor of apoptosis and cell proliferation (Vousden & Lu, 2002), could contribute to the ability of detached cells that did not succumb to ErbB2 loss (Figure 33a, 34b) to survive and grow in the dense cell culture.

We further observed that the number of cells grown in the sparse culture (and producing relatively high ErbB2 amounts) was significantly reduced by trastuzumab treatment (Figure 34c). We also found that treatment with selumetinib (which downregulates ErbB2 in these sells (Figure 28b) reduced the number of detached cells to a degree similar to that observed in case of trastuzumab treatment (Figure 34c). These data are consistent with a notion that has emerged from our studies (Figure 28-30) that Mek inhibition blocks anchorage-independent growth of breast cancer cells at least in part by downregulating ErbB2. We further noticed that a combination of selumetinib and trastuzumab had the same impact on the growth of detached cells as each drug alone (Figure 34c). The latter data support a scenario that once selumetinib blocks ErbB2 expression in the cells, trastuzumab (that no longer has a target) does not affect growth of those cells that are still present in the culture after selumetinib treatment.

Remarkably, trastuzumab treatment failed to reduce the number of ErbB2-deficient cells that were present in the dense culture (Figure 34d). In addition,

selumetinib or its combination with trastuzumab did not reduce the number of detached densely grown cells (Figure 34d). Thus, increased density of detached breast cancer cells strongly reduces Erk, phospho-Erk and ErbB2 levels in these cells and renders those cells that remain viable trastuzumab-resistant.

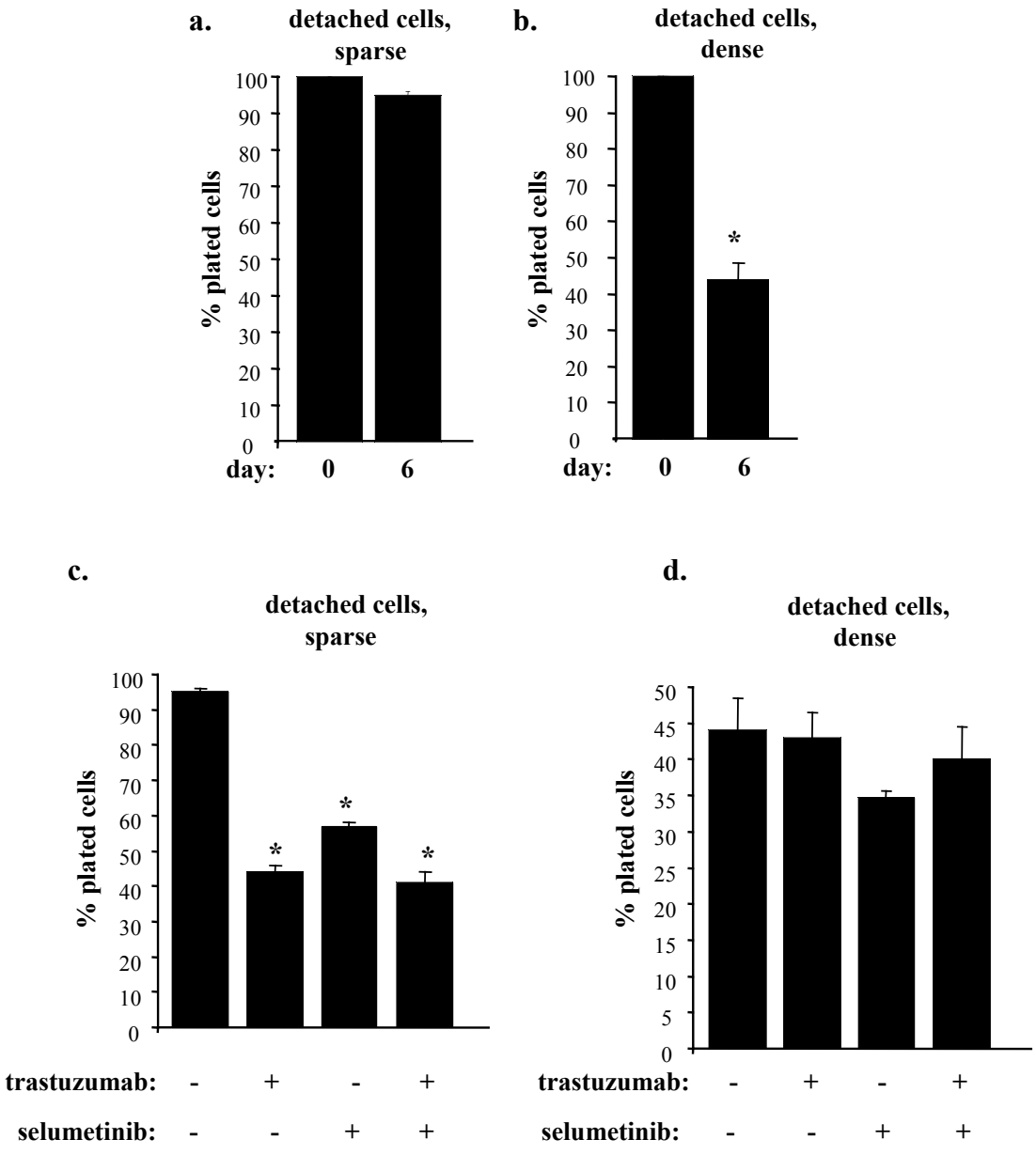

Figure 34. Trastuzumab sensitivity is blocked by an increased density of detached breast tumour cells. (a, b). BT-474 cells were cultured detached from the ECM for 6 days at a concentration 12500 cells/ml (sparse) (a) or 125000 cells/ml (dense) (b) for 6 days and the cells were counted. (c, d). BT-474 cells were cultured detached from the ECM for 6 days at a concentration 12500 cells/ml (sparse) (c) or 125000 cells/ml (dense) (d) for 6 days in the presence of DMSO (-) or 1μM selumetinib (+) or 5μg/ml

trastuzumab (+) and the cells were counted. % plated cells were calculated as that of the cells plated at day 0. The data represent the average of the triplicates (a, b, d) or that of the duplicates (c) plus SD. * indicates that p value was < 0.05.

3.3.6 Summary

In summary, we have identified a novel mechanism of the regulation of ErbB2 expression in breast cancer cells. When the cells detach from the ECM this expression becomes strongly dependent on Mek activity. Furthermore, the ability of Mek to promote anchorage- independent growth of such cells requires Mek-induced ErbB2 upregulation. Once Mek activity is blocked in breast cancer cells, the cells lose ErbB2 and those cells that survive become trastuzumab-resistant.

CHAPTER 4. DISCUSSION

The text of the sections 4.1 - 4.5 and 4.12 - 4.16 are verbatim as they have been published by us (I. A. Khan et al., 2016) and (Iman A. Khan et al., 2014) respectively. The text of the sections 4.6 – 4.9 and 4.11 are verbatim of our submitted manuscript (Iman Aftab Khan, 2018).

4.1 Perp is a mediator of ErbB2-dependent malignant transformation of breast cancer cells.

We have identified here a novel mechanism by which ErbB2 oncoprotein blocks anoikis of breast epithelial cells. This mechanism is driven by ErbB2-induced downregulation of a pro-apoptotic protein Perp. Since anoikis resistance is thought to be critical for breast cancer progression (Debnath & Brugge, 2005; Debnath et al., 2002) mechanisms that block Perp expression in breast tumour cells represent potential new targets for breast cancer treatment.

Our data are relevant to human breast cancer as Perp expression is frequently reduced in human breast cancer cells compared to the non-malignant breast epithelial cells (Dusek et al., 2012). Moreover, deletion of one Perp allele in the mammary tissue of mice lacking p53 tumour suppressor gene significantly accelerates breast tumour growth in these mice and substantially reduces their tumour-free survival (Dusek et al., 2012). Perp deficiency has also been demonstrated to enhance presence of inflammatory cells in the mouse mammary epithelium (Dusek et al., 2012). Inflammation plays an important role in invasive transformation of cancer cells during metastasis (Kitamura, Qian, &

Pollard, 2015). Therefore, in addition to promoting anoikis resistance of ErbB2-positive breast cancer cells, loss of Perp may also promote breast tumour growth by inducing inflammatory response.

In further support of the important role of Perp in epithelial cancer progression it was found that Perp ablation in the mouse epidermis significantly enhances the formation of UV-induced squamous skin cell carcinomas in mice (Beaudry et al., 2010). It was also found that Perp downregulation in tumours of patients with oral cavity squamous cell carcinomas correlates with the transition from oral dysplasia to invasive carcinoma and further to metastatic disease (Kong et al., 2013). Loss of Perp was also observed to correlate with increased chances of relapse of these malignancies (Kong et al., 2013). Of note, ErbB2 expression (which as we found causes Perp loss in breast cancer cells) is elevated in a significant fraction of human oral squamous cell carcinomas (A. J. Khan et al., 2002; W. Xia et al., 1997).

4.2 Perp is an inducer of anoikis.

Perp is a component of the desmosomes, and so far, its known physiological roles have been limited to the regulation of cell-to-cell contact in various contexts (Beaudry et al., 2010; Ihrie et al., 2005). In this study we identified a novel physiological role for Perp: we demonstrated that Perp is a mediator of anoikis of the non-malignant breast epithelial cells. We found that when these cells detach from the ECM, Perp is upregulated, and this contributes to their anoikis. The ability of Perp to promote apoptosis of detached cells is consistent with observations that several types of cells derived from Perp-deficient mice are significantly less susceptible to apoptosis induced by various

stimuli than the respective control cells derived from the wild type mice (Ihrie et al., 2003) (i.e. the ability of Perp to cause apoptosis is well established). Whether or not Perp-mediated anoikis is related to the involvement of this protein in the formation of the desmosomes is the subject of our ongoing research. The fact that Perp blocks cell growth in soft agar (Figure 13d) when the cells are placed in agar a single cell 'suspension' in which the cell-to-cell contacts are not allowed to be formed is consistent with a scenario that Perp kills them via desmosome-unrelated mechanisms.

4.3 Perp is a mediator of EGFR signalling.

In this study we identified the mechanism of detachment-induced Perp upregulation in the non-malignant breast epithelial cells. We found that this upregulation is triggered by detachment-induced EGFR loss. These studies are consistent with our previous findings indicating that EGFR activation blocks anoikis of intestinal epithelial cells (Rosen et al., 2001). Others made similar observations in case of skin (Jost et al., 2001), kidney (Schulze et al., 2001) and breast (M. J. Reginato et al., 2003) epithelial cells.

4.4 Perp-dependent pro-anoikis mechanisms are ErbB2 targets.

We demonstrated here that ErbB2 blocks anoikis of breast epithelial cells by preventing detachment-induced Perp upregulation. Thus, ErbB2 rescues detached cells from apoptosis by disrupting cellular anoikis-promoting machinery. We found previously that Ras, another major oncoprotein, also blocks anoikis by inactivating such machinery in intestinal epithelial cells. We observed that anoikis of these cells is caused in part by the downregulation of the anti-apoptotic protein Bcl-X_L induced by detachment of these

cells from the ECM. We found that Ras suppresses their anoikis by preventing detachment-dependent Bcl-X_L downregulation (K. Rosen et al., 2000). Hence, inactivation of the anoikis-promoting events appears to be one of the mechanisms by which oncoproteins allow cancer cells to grow as three-dimensional masses.

4.5 Perp is a mediator of the MAP kinase signalling.

We found in this study that ErbB2 prevents detachment-induced Perp upregulation in a manner that requires the activity of a protein kinase Mek. These results are consistent with observations made by us and others that ErbB2 blocks detachment-induced loss of EGFR (and this loss causes Perp upregulation) via Mek-dependent mechanisms (Grassian, Schafer, et al., 2011). It is likely that once detachment-induced EGFR downregulation is blocked, EGFR can homodimerize or heterodimerize with other ErbB family members (Harari & Yarden, 2000) to downregulate Perp in the detached cells.

A regulatory protein Sprouty-2 can act both as a positive as well as negative regulator of Erk signaling (G. R. Guy, Jackson, Yusoff, & Chow, 2009; Wong et al., 2002). Binding of human sprouty-2 to wild-type Raf kinase is known to inhibit active Raf kinase in the presence of fibroblast growth factor ligand (Tsavachidou et al., 2004; Yusoff et al., 2002) and potentiates Erk signaling by preventing EGFR ubiquitylation and endocytosis in the presence of EGF ligand (Wong et al., 2002). Sprouty-2 dependent upregulation of EGFR was found to promote survival of detached breast epithelial cells (Grassian, Schafer, et al., 2011). Our data are consistent with the latter scenario in that it demonstrates that survival of detached breast epithelial cells depends upon Sprouty-2-

induced EGFR upregulation followed by EGFR-mediated inhibition of Perp expression (see Chapter 3). Hence Sprouty-2 appears to play an oncogenic role in our model system. In agreement with our data, it has been observed that glioblastoma cells overexpress Sprouty-2 and EGFR (Walsh et al., 2015). Moreover, inhibition of Sprouty-2 was found to suppress anchorage-independent growth and tumorigenicity of glioblastoma cells in mice (Walsh et al., 2015). Furthermore, stable knockdown of Sprouty-2 in the presence of EGFR inhibitors was found to enhance death of the indicated cells (Walsh et al., 2015). In addition, Sprouty-2 was observed to be highly expressed in the invasive colon carcinoma cells (Holgren et al., 2010).

4.6 Irf6 is downregulated by ErbB2 in breast cancer cells.

We have identified here a novel mechanism of ErbB2-dependent inhibition of anoikis of breast epithelial cells. This mechanism involves ErbB2-dependent downregulation of a transcription factor Irf6. Our results are relevant to breast cancer as, according to what was published by others, a significant fraction of human breast cancers tends to produce relatively low Irf6 levels (Uhlen et al., 2005). Moreover, we have demonstrated that all locally advanced ErbB2-positive breast cancers studied by us are essentially Irf6-negative (these data were obtained by our collaborators and are included in the manuscript recently submitted by us (Iman Aftab Khan, 2018)). Breast cancer is not the only malignancy associated with Irf6 downregulation: such downregulation also occurs in nasopharyngeal and squamous cell carcinomas (Botti et al., 2011; Xu et al., 2017).

4.7 Irf6 as an inducer of anoikis.

Others found that Irf6 is upregulated in the breast during mammary gland involution upon cessation of lactation (Bailey et al., 2009). This involution is accompanied by the production of ECM-degrading proteases by the mammary gland and is likely, at least in part, mediated by anoikis of breast epithelial cells (Jane Wiesen & Zena Werb, 2000). The possibility that Irf6 mediates such anoikis is supported by our data. We found that when non-malignant breast epithelial cells detach from the ECM, Irf6 is upregulated, and contributes to apoptosis of these cells. The ability of Irf6 to promote apoptosis is consistent with what was published by others. For example, it was observed that Irf6 knockdown by RNAi reduced apoptosis of neurons triggered by a traumatic injury (Y. Lin et al., 2016).

4.8 Detachment-induced Irf6 upregulation requires the presence of a transcription factor ΔNp63α.

We have shown that detachment-induced Irf6 upregulation in non-malignant breast epithelial cells requires the presence of a transcription factor ΔNp63α, a transcription factor that was found by others to directly control Irf6 gene transcription (Moretti et al., 2010). Perhaps not by coincidence, ΔNp63α as well as other p63 isoforms are typically not produced by breast cancer cells (Como et al., 2002).

4.9 ErbB2 blocks the expression of multiple Irf6 target mRNAs that encode mediators of apoptosis.

We have found that ErbB2 alters the expression of mRNAs encoding numerous Irf6 targets that control apoptosis. We have observed that ErbB2 upregulates an mRNA encoding an apoptosis inhibitor Tenascin-C (TNC) (Shi et al., 2015) and downregulates the mRNAs encoding pro-apoptotic proteins, such as keratin 23 (KRT23) (Birkenkamp-Demtroder et al., 2007), B-cell linker (BLNK) (Nakayama et al., 2009), N-Myc downstream-regulated gene 2 (NDRG2) (Park et al., 2018), protease serine S1 family member 8 (PRSS8) (L. Zhang et al., 2016), tumour-associated calcium signal transducer 2 (TACSTD2) (Wang et al., 2014), early growth response gene (EGR3) (Xi & Kersh, 2004), sphingosine-1-phosphate phosphatase 2 (SGPP2) (W.-C. C. Huang et al., 2016), Ras association domain family member 5 (RASSF5) (X.-H. H. Zhou et al., 2014) and Zinc Finger Protein 36 (Zfp36) (Selmi et al., 2015). Thus, it is likely that ErbB2 blocks anoikis of breast cancer cells by inhibiting Irf6-dependent pro-apoptotic program controlled by a complex network of mediators of programmed cell death. Establishing which of these proteins control anoikis downstream of Irf6 and how they functionally interact with each other and with the direct regulators of the cellular apoptotic machinery (i.e. the Bcl-2 family proteins, caspases etc.) represents an important direction for our future studies.

4.10 Perp is a mediator of Irf6 signalling in breast epithelial cells.

We found that detachment-induced upregulation of Perp in breast epithelial cells requires the presence of Irf6 (Fig. 27a). Moreover, we observed that expression of the

exogenous Irf6 in the attached breast epithelial cells mimics the effect of detachment on Perp in that Irf6 upregulates Perp in the attached cells (Fig. 27c). Thus, Perp is a mediator of Irf6 signalling in breast epithelial cells. Transcription factors often bind consensus DNA sites or those complementary to them. A sequence closely resembling the one complementary to the Irf6 consensus binding site (Botti et al., 2011) is present in the Perp gene from multiple species (Figure 26). Hence it is possible that Irf6 upregulates Perp in detached breast epithelial cells by directly binding to the Perp promoter. Alternatively, Irf6 could upregulate Perp indirectly. Investigating the precise mechanisms by which Irf6 controls Perp expression represents an interesting direction of future studies aimed at understanding how ErbB2 regulates anoikis of breast cancer.

Another important direction of these studies would be to find out whether ErbB2-induced downregulation of Perp in breast tumour cells is the consequence of ErbB2-dependent Irf6 downregulation.

4.11 ErbB2-targeted drugs block the effect of ErbB2 on Irf6.

ErbB2-driven anoikis resistance of breast tumour cells is thought to be a prerequisite for breast cancer progression (Debnath & Brugge, 2005; Debnath et al., 2002). Thus, drugs that upregulate Irf6 in breast tumour cells can be expected to block or attenuate the disease. Noteworthy, we found that trastuzumab, an anti-Erbb2 therapeutic antibody (Slamon et al., 2001) and lapatinib, a small molecule ErbB2/EGFR used for treatment of ErbB2-positive breast cancers (Geyer et al., 2006), upregulate Irf6 in ErbB2-producing human breast cancer cells in tissue culture. Moreover, we demonstrated that neoadjuvant trastuzumab-based treatment of patients with locally advanced breast cancer

appears to be associated with an increase in Irf6 levels in respective tumours derived from a pilot patient cohort (these data were obtained by our collaborators and are included in the manuscript recently submitted by us (Iman Aftab Khan, 2018)). These data are consistent with the possibility that ErbB2 controls Irf6 levels in patients' tumours.

4.12 Mek activity is required for ErbB2 expression in breast cancer cells detached from the ECM

We have identified in this study a novel mechanism by which a protein kinase Mek controls three-dimensional growth of ErbB2-positive breast cancer cells. Mek promotes growth of these cells without adhesion to the ECM by supporting ErbB2 expression. This function of Mek is somewhat unexpected. It is well known that the indicated protein kinase can promote Erk-dependent phosphorylation and thus alter the activity of numerous transcription factors (Yoon & Seger, 2009). One easily conceivable scenario for the role of Mek in anchorage-independent growth of tumour cells is that once activated downstream of ErbB2, Mek alters the activity of those transcription factors that directly control the expression of the regulators of cell growth and survival and thus promotes growth of these cells without adhesion to the ECM. However, this is not the only manner in which Mek can mediate ErbB2 signalling. We found that the ability of breast epithelial cells producing an activated Mek mutant to grow anchorage-independently is substantially impaired if the activity of ErbB2 (which is strongly upregulated by Mek in these cells) is blocked by trastuzumab (Figure 30). Hence three-dimensional growth of these cells significantly relies on the ability of Mek to upregulate

ErbB2, rather than (or in addition to) the ability of Mek to promote phosphorylation of transcription factors that directly control levels of proteins regulating cells growth and survival. We found that ErbB2 blocks anoikis of breast cancer cells by downregulating pro-apoptotic proteins Perp (I. A. Khan et al., 2016) and Irf6 in a Mek-dependent manner (Iman Aftab Khan, 2018). It would be of significant interest to test whether the effect of Mek on these proteins requires Mek-driven ErbB2 upregulation in the indicated cells.

4.13 Mek blocks lysosomal degradation of ErbB2

Our data are consistent with a scenario that Mek promotes ErbB2 expression in breast cancer cells by blocking lysosomal ErbB2 degradation. These data are supported by findings that ErbB2 can be degraded by the lysosomes in breast cancer cells in response to various stimuli. For example, it was established that a therapeutic HSP90 antagonist can trigger lysosomal ErbB2 degradation in breast cancer cells (see section 1.12 page 73) (Castagnola et al., 2014). Moreover, an inhibitor of phosphatidylcholine-specific phospholipase C can promote lysosomal ErbB2 degradation in these cells as well (see section 1.12 page 73) (Paris et al., 2010). A similar mode of regulation was identified for the ErbB2 signalling partner EGFR. It was found that detachment of non-malignant breast cancer cells promotes lysosomal EGFR degradation (Grassian, Schafer, et al., 2011). Perhaps not by coincidence, it was observed that ErbB2 blocks lysosomal EGFR degradation by activating Mek (Grassian, Schafer, et al., 2011).

4.14 Changes in Mek activity is a possible cause of tumour heterogeneity with regard to ErbB2 expression.

It is known that ErbB2 levels are not constant in many ErbB2-positive breast cancers. ErbB2 protein expression is heterogeneous (i.e. some parts of the tumour produce ErbB2 and some do not) in a significant fraction of ErbB2-positive breast cancers that display a relatively homogeneous amplification of the ErbB2 gene (the latter is normally detected by Fluorescence In Situ Hybridization (FISH)) (Kurozumi et al., 2016). Importantly, patients with heterogeneous ErbB2 expression in breast tumours have a worse prognosis than those with a homogeneous ErbB2 expression (M. J. Reginato et al., 2003). What factors control this heterogeneity is not known. Our data indicate that the activity of Mek or that of its effectors could be responsible for this phenomenon. Our results suggest that tumour cells with lower Mek activity produce lower ErbB2 amounts than those with a higher Mek activity.

4.15 Mek activity and ErbB2 expression are controlled by tumour cell density.

We found that an increase in the cell density within a three-dimensionally grown mass of breast cancer cells strongly blocks the expression and phosphorylation of the Mek substrate Erk and essentially eliminates ErbB2 from the cells (Figure 33a). (The mechanisms by which Erk expression is reduced under these conditions are presently not known and represent an important direction for our future studies). These data are consistent with observations that growth of tumours composed of ErbB2-positive breast cancer cells injected in immunodeficient mice is suppressed by trastuzumab much more

efficiently if the drug is administered immediately after cell injection (before the tumour mass is formed) compared to the scenario when the drug is administered after a tumour mass is established (Ithimakin et al., 2013). Situations when a change in the breast tumour cell density could take place in breast cancer patients and thus affect ErbB2 levels have been described (Wülfing et al., 2006). For example, it was found that circulating breast cancer cells (that are likely relatively sparse) are ErbB2-positive in a substantial number of cases while primary tumour masses (in which tumour cell density is likely higher than that in case of the circulating tumour cells) derived from the same patients are often ErbB2-negative (Wülfing et al., 2006). Testing whether the circulating tumour cells display higher phospho-Erk levels than those composing respective primary ErbB2-negative tumours would represent a promising direction of the studies aimed at verifying the role of Mek/Erk activity in ErbB2 expression in breast cancer patients.

4.16 Variable Mek activity and ErbB2 expression as potential causes for variable sensitivity of breast cancers to ErbB2-targeted drugs.

Primary tumours showing equivocal results of the immunohistochemical measurement of the ErbB2 levels are normally tested for ErbB2 gene amplification by FISH (H. Lee et al., 2014). If the ErbB2 gene is amplified, respective patients receive trastuzumab-based therapies even though their tumours do not display high levels of ErbB2, and the indicated treatments can provide benefit to these patients (H. Lee et al., 2014). The reasons why such therapies may be effective in such cases are unclear. Our study provides a potential mechanistic explanation of these observations. Respective primary tumours carrying an amplified ErbB2 gene might display low ErbB2 levels due

to low Mek/Erk activity. However, cancer cells remaining in the body after the primary tumour is resected could have a higher Mek/Erk activity and ErbB2 expression than the cells in the primary tumour and thus be at least partially sensitive to subsequent trastuzumab-based treatments.

4.17 Future directions

We have demonstrated that ErbB2 promotes three-dimensional growth of breast cancer cells in tissue culture by downregulating Perp and Irf6. One important direction for future studies would be to test whether the effect of ErbB2 on these proteins contributes to the ability of malignant cells to form tumours and metastases in mice. It will also be of interest to establish to what degree upregulation of Perp and Irf6 by ErbB2-targeted drugs contributes to the effect of ErbB2-directed therapies. One approach to addressing this question would be to knock down Perp or Irf6 in ErbB2-overexpressing breast cancer cell and test how Perp or Irf6 loss affects sensitivity of these cells to the indicated drugs.

Of note, RNA sequencing data indicate that most ErbB2-positive tumours have lower Irf6 and Perp mRNA levels than a significant fraction of ErbB2-negative tumour (Botti et al., 2011; Uhlen et al., 2005; Xu et al., 2017). Remarkably, no mortality was observed among a minority (5-10%) of patients with ErbB2-positive primary tumours showing relatively high Perp (Botti et al., 2011; Uhlen et al., 2005; Xu et al., 2017) or Irf6 (Uhlen et al., 2005) mRNA levels (possibly because ErbB2 failed to downregulate Perp or Irf6 in these tumours). In contrast, the mortality rate of patients in whose ErbB2-positve tumours Perp or Irf6 mRNAs levels were low was 10-15% (Botti et al., 2011;

Uhlen et al., 2005; Xu et al., 2017). Thus, low levels of Irf6 and Perp mRNAs in ErbB2-positive breast tumours signify decreased patient survival. Thus, it is possible that baseline levels of Perp and Irf6 expression in breast cancer cells could be used to predict the survival of patients with ErbB2-positive tumours.

It is also worth mentioning that patients with ErbB2-positive, locally advanced breast cancer normally receive neoadjuvant trastuzumab and chemotherapy for approximately 3 months followed by tumour resection and further trastuzumab therapy for approximately 9 months. Cancer relapses in about 30% patients (Untch et al., 2011). Whether the relapse will occur cannot be predicted reliably at present. Trastuzumab can cause serious side effects (e.g. cardiotoxicity (Telli, Hunt, Carlson, & Guardino, 2007)) and is costly (Drucker et al.). Thus, predicting which patients can benefit from the drug could allow potential non-responders to avoid side effects of unwarranted therapy. However, predictive markers of patients' response to trastuzumab are not available. We found in this study that ErbB2 promotes three-dimensional growth of breast cancer cells by downregulating ΔNp63α, Irf6 and Perp as well as by upregulating EGFR and Sprouty 2. Testing whether trastuzumab-induced upregulation of ΔNp63α and/or Irf6 and/or Perp and/or trastuzumab-dependent downregulation of EGFR and/or Sprouty 2 in patient tumours after the initial 3-months treatment could be used to predict whether respective patients will benefit from the subsequent 9-month trastuzumab-based therapy.

A significant fraction of ErbB2-positive breast cancers develops resistance to ErbB2-directed therapies. Since ErbB2-dependent downregulation of Perp and Irf6 significantly contributes to the ability of breast cancer cells to grow in a three-

dimensional manner molecular mechanism by which ErbB2 downregulates these proteins represent potential novel targets for treatment of ErbB2-positve breast cancers.

We have demonstrated that the effect of ErbB2 on Irf6 and Perp in breast cancer cells can be blocked by Mek and Erk inhibitors. Such inhibitors could in principle be used for treatment of ErbB2-positive breast cancers. Noteworthy, one of the Mek inhibitors did not show promising results in a breast-cancer-directed clinical trial (although the effect of the drug on ErbB2-positve cancers was not investigated). One likely reason for these results is that Mek inhibitors are known to activate various feedback mechanisms (e.g. ErbB3 upregulation) which trigger various oncogenic signalling events in cancer cells (Morris et al., 2013; C. Sun et al., 2014). In contrast, a small molecule Erk inhibitor SCH772984 (which according to our studies upregulates Irf6 in ErbB2-positve breast epithelial cells) was recently demonstrated to efficiently block MAP kinase-dependent growth of tumour cells without triggering any feedback events (Hayes et al., 2016). Testing whether such drugs are effective against ErbB2-positive breast cancers resistant to ErbB2 antagonists and whether the effect of these Erk inhibitors on tumour cells is mediated by Irf6 and/or Perp represents a promising direction for future studies.

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APPENDIX A. AUTHOR CONTRIBUTIONS

I would like to profusely acknowledge the contributions made by the co-authors of all the manuscripts mentioned in this thesis.

For the published manuscript (I. A. Khan et al., 2016) *"ErbB2-dependent downregulation of a pro-apoptotic protein Perp is required for oncogenic transformation of breast epithelial cells."* I would like to thank Dr. Byong Hoon Yoo for performing the experiment shown in Figure 5 (c) (data not shown in the thesis) presented in the original manuscript. Dr. Olivier Masson had prepared the samples for microarray analysis. Dale Corkery, a PhD candidate in Dr. Graham Dellaire's lab (Department of pathology, Dalhousie University, Halifax, NS, Canada) performed the experiment with ovarian cancer cells shown in Figure 11(i) on page 62 in this thesis. Sylvain Baron from Dr. Laura D. Attardi's lab (Department of Radiation Oncology and Cancer Biology, Stanford University, CA, USA) had provided us with the pLex vectors and the packaging plasmids in order to perform experiments shown in the Figure 13(c) on page 66 in this thesis.

For the submitted manuscript *"ErbB2-driven downregulation of a transcription factor Irf6 in breast epithelial cells is required for their three-dimensional growth."* I would like to acknowledge the contributions made by Dr. Byong Hoon Yoo that were crucial for this paper. He had generated the vectors: pBabe-HA-Irf6 and pBabe-ΔNp63α-FLAG for this study and had performed experiments shown in Figure 21 (c-e) and 25 (d) on pages 83 and 91 respectively of this thesis. I am also thankful to our collaborators Dr. Gillian Bethune and colleagues from the Department of Pathology, Dalhousie University,

Halifax, NS, Canada for performing the immunohistochemistry experiment shown in Figure 9 (data not shown in this thesis) of the original manuscript.

For the published manuscript (Iman A. Khan et al., 2014) *"Mek activity is required for ErbB2 expression in breast cancer cells detached from the extracellular matrix."* I would like to thank Dr. Byong Hoon Yoo for performing the experiments shown in Figure 5 d-e (data not shown in the thesis) of the original manuscript. He also quantified all the western blots and generated figures shown in supplementary figure 1-4, 5 (b) and 6 (data not shown in the thesis). I would also like to thank our collaborator Dr. Janusz Rak, Department of Pediatrics, McGill University, Montreal, Canada for his valuable suggestions in designing the experiment and presentation of the data shown in Figure 33 on page 108 of this thesis.

Last but not the least, I am truly thankful to Dr. Mauricio Reginato, Department of Biochemistry and Molecular Biology, Drexel University, PA, USA for providing us with breast epithelial cell lines MCF10A and its derivatives for this study. Dr. Stadnyk, Department of Microbiology and Immunology, Dalhousie University, Halifax, NS, Canada provided us with the Hek293T cells required for generating viral preps. Francesca Moretti from Dr. Antonio Costanzo's lab, Department of Neuroscience, University of Rome, Italy for providing us with pcDNA-HA-Irf6 vector.

APPENDIX B. SUPPLEMENTARY MATERIAL

Table 1 . Pharmacological compounds and Concentrations.

Pharmacological compounds	Company	Catalogue no.#	Final concentration
Lapatinib Dictosylate	Selleckchem	S1028	1 μM
SCH772984	Selleckchem	S7101	1 μM
PD98059	Sigma	P215	25 μM
LY294002	Sigma	L9908	20 μM
Selumetinib	SantaCruz or ApexBio	sc-364613 or A8207	1 μM
Bafilomycin A1	Sigma	B1793	100 nm
MG-132	Calbiochem	474790	10 μg/ml
Cycloheximide	Sigma	C7698	10 μg/ml
Trastuzumab	Roche	DIN 02240692	5 μg/ml
Polybrene	Sigma	TR-1003-G	8 μg/ml

Table 2. Cell culture medium and Reagents.

Cell culture medium and reagents	Company	Catalogue no.#
DMEM	Gibco	12800-82
DMEM-F12	Gibco	12500-062
RPMI-1640	Gibco	31600-022
Hybri-care medium	ATCC	46-X
McCoy's-5a medium	Gibco	16600-082
MEBM and MEGM	Lonza	CC-3151
EGF	Invitrogen	PHG0311
Insulin	Novolin GE Toronto	DIN 02024233
Hydrocortisone	Sigma	H0135
Cholera toxin	Biolynx, Inc	ENZ-G117-0001
Horse serum	Gibco	16050-122
Fetabl Bovine Serum	Seradigm	1500-500
Penicillin-Streptomycin	Gibco	15140-122
L-Glutamine	Gibco	25030-081
Hygromycin-B	Invitrogen	10687-010

Cell culture medium and reagents	Company	Catalogue no.#
Zeocin	Invitrogen	R250-01
Puromycin	Gibco	A11138-03
Lipofectamine2000	Invitrogen	11668-019
Opti-MEM	Gibco	31985-070
Bacto-Agar	Becton Dickinson (BD) and Company	214010
Sea-plaque agarose	Lonza	50100
Protease inhibitor cocktail	Roche Diagnostics	11697498001
Apoptosis Detection Kit	Millipore	APT750
sodium vanadate	Sigma	S6508
PMSF	Sigma	P7626
Ammonium persulfate	Sigma	A3678
Immobilon-P PVDF	Millipore	IPVH00010
Bicinchoninic protein assay kit	Thermo Scientific	23227
Bovine serum albumin	Sigma	A9647
Enhanced Chemiluminescence reagent	GE healthcare or Millipore	RPN2106 or P90720
X-ray films	Mandel	XC6A2
30% Acrylamide/Bis Solution 37.5:1	Bio-Rad	1610158
mRNA extraction kit	Qiagen	74134
Reverse Transcription Kit	Clontech	639549
Tetramethylethylenediamine	Bio-Rad	1610801
Taq DNA polymerase	Invitrogen	11304-011
T4 DNA ligase buffer	New England Biolabs	B0202S
Hoescht stain	Molecular Probes	H1398
N, N-bis (2 hydroxyethyl)-2- aminoethanesulfonic acid	Sigma	B4554
BamH1-HF	New England Biolabs	R3136S
SalI-HF	New England Biolabs	R0138S
DNA ladder	Invitrogen	10787-018
Agarose	Bio-Rad	161-3101
QIAquick Gel Extraction Kit	Qiagen	28704

Table 3. Antibodies Information.

Antibodies	Company	Catalogue no. #	Final dilution
Anti-Perp	Abcam	ab5986	1:1000
Anti-Sprouty2	Abcam	ab60719	1:500
Anti-IRF6	Cell Signalling	6948	1:1000
Anti-ErbB2/Her2	Cell Signalling	2242	1:6000
Anti-Akt1	Cell Signalling	2967	1:1000
anti-phospho-Akt	Cell Signalling	4060	1:1000
Anti-MAPK/Erk	Cell Signalling	9102	1:1000
Anti-phospho-MAPK/Erk	Cell Signalling	9101	1:1000
Anti-Mek1/2	Cell Signalling	9122	1:1000
Anti-Phospho-Mek1/2	Cell Signalling	9121	1:1000
Anti-GAPDH	Cell Signalling	2118	1:60000
Anti-HA Tag	Cell Signalling	2367	1:1000
Anti-β-actin	Sigma and Santa Cruz Biotechnology	A5441 or sc-130656	1:500000
Anti-RSK	Cell signalling	9355	1:1000
Anti-phospho-RSK	Cell Signalling	9344	1:1000
Anti-EGFR	Santa Cruz Biotechnology	sc-03	1:1000
Anti-CDK4	Santa Cruz Biotechnology	sc-601	1:1000
Anti-**Δ**Np63	Biolegend	619002	1:1000
Anti-tGFP	OriGene Technologies	TA150041	1:1000
Anti-Rabbit IgG	Bio-Rad	170-6515	1:3000
Anti-Mouse IgG	Bio-Rad	170-6516	1:5000

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Title: ErbB2-dependent downregulation of a pro-apoptotic protein Perp is required for oncogenic transformation of breast epithelial cells

Author: I A Khan, B H Yoo, O Masson, S Baron, D Corkery et al.

Publication: Oncogene

Publisher: Springer Nature

Date: Apr 25, 2016

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