Translational Control of Stress Induced Breast Cancer Plasticity

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Abstract

Cancer cell plasticity, whereby stem-cell-like properties are acquired in response to stress, represents a major challenge in cancer treatment. Using cell line and patient-derived xenograft (PDX) models, we demonstrate that translational reprogramming occurs in response to stresses such as hypoxia and chemotherapy which elicits stem cell properties in breast cancer. Other stresses like chemotherapy demonstrate the translational inhibition that has been well characterized as an integral hypoxic cellular response. The inhibition of translation is primarily regulated at initiation by mTOR/4E-BP1 and PERK/eIF2α signalling. We identify previously undescribed 5’ untranslated regions (5’UTRs) of plasticity-associated genes like NANOG, SNAIL and NODAL. A subset of these isoforms facilitates efficient translation during times of stress when global protein synthesis is reduced. We demonstrate that these mRNAs are translationally upregulated by mTOR suppression and concomitant up-regulation in eIF2α phosphorylation, and that translation underpins the reprogramming of breast cancer stem cell (CSC) phenotypes such as self-renewal and epithelial-mesenchymal transition (EMT). Accordingly, we demonstrate that mTOR inhibitors induce stem cell phenotypes in breast cancer, partially explaining their lower-than-expected clinical efficacy. Most notably, we report that stress-induced CSC phenotypes can be overcome with drugs that antagonize the effects of eIF2α phosphorylation and increase ternary complex recycling (e.g. ISRIB). Indeed, studies conducted using cell lines and PDX models indicate that ISRIB decreases CSCs in hypoxic breast tumours and increases the efficacy of mTOR inhibitors and chemotherapies by suppressing breast cancer plasticity.

Keywords

Translation, mTOR, Unfolded Protein Response (UPR), Integrated Stress Response, Nodal, ISRIB, Polysome, 5’Untranslated Region, PDX, Cancer Stem Cells, uORF, Epithelial to Mesenchymal transition, Plasticity, Hypoxia
Co-Authorship Statement

Michael Jewer conducted western blots, RT-PCR assays, cloning, reporter assays, sphere formation assays, colony formation, IHC analysis and optimization, viability assays, flow cytometry and transfections. Laura Lee completed the RNAseq analysis and quantification of necrosis. Guihua Zhang and Jiahui Liu performed all animal experiments and IHC. Scott Findlay and Michael Jewer designed isoform-specific PCR assays and cloned constructs for reporter assays; Krista M. Vincent and Michael Jewer conducted analyses of patient data. Indrani Dutta assisted with flow cytometry. Mackenzie Coatham assisted RT-PCR of stem cell genes. Andrea Brumwell and James Uniacke assisted with polysome fractionation and Christos Patsis and Antonis Koromilas provided the eIF2α KI expressing cells. Julia Schueler supplied PDX models, Gabrielle M. Siegers assisted, flow cytometry and characterization of PDX models.
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Chapter 1

1 General Introduction and Literature Review

Cancer is a multi-etiologic disease with a complex progression and various presentations. Even with the dramatic advancements in treatment options, it remains the second leading global cause of death [1]. Currently, nearly 1 in 6 deaths are the result of cancer. These deaths represent enormous personal costs to individuals and families affected by the disease. In addition to these tragedies, there is a vast economic cost, in excess of 1.16 trillion dollars per annum [2]. Of the multitude of cancers, breast cancer is the second leading cause of death of women in Canada. Astonishingly, breast cancer diagnoses will account for a quarter of all new cancers in 2018 [3]. Due to improvements in screening and treatment, these breast cancer diagnoses make up only 13% of cancer-related deaths. Though the incident rate has fluctuated over the last 30 years, the death rate has been steadily declining since the mid-1980s [3]. Cancer survivorship rates in many parts of the world are the best they have ever been, but due to the evolving and progressive nature of the disease many people still lose their lives to cancer and cancer-related complications.

1.1 A History of Heterogeneity and Cancer

For decades physicians and scientists have been observing and attempting to understand cancer. Many early observers have contributed to our appreciation of this complex disease. A profound example of this was in 1838 when Johannes Muller published his observations in the form of nearly 100 gross and microscopic drawings, wherein he described cancer as groupings of abnormal cells, noted changes in the stroma, and identified their growth and metastasis to specific organs [4, 5]. He further deemed these cells to be destructive and capable of vascular travel. Muller also noted the histological differences between numerous tumour types and the role of necrosis in tumour regression [4, 5]. From some of our earliest observations, we have known that cancer is an incredibly diverse, complex and deadly disease.
1.1.1 Describing Heterogeneity Through Grading and Staging Techniques

A profoundly useful tool in grappling with the complexity of cancer has been the description and classification of different subcategories of each disease [6]. Currently grading, staging, histological analysis, and protein expression act as surrogates for a collection of phenotypes and molecular networks underpinning each disease [7]. For decades these subtypes have been used to test and predict responses to treatments, allowing primary care providers to treat patients more efficaciously [7].

Though our schema of tumour complexity has become more sophisticated, research is only starting to truly grasp the extent of the heterogeneity of different cancers, individual tumours of the same cancer, and even subsets of cells within a single tumour. Starting from simple metrics like tumour growth rate, we observe an incredible diversity of phenotypes. An analysis of 395,188 women whose mammograms were measured to estimate changes in tumour volume demonstrated that the mean doubling time of breast cancer tumours ranged from 41 days in the first quartile to 234 for the last, with a much greater amount of time between the fastest and slowest growing tumours [8]. Knowing that a metric as simple as tumour size can help predict the efficacy of treatment and recovery of patients, the notion that other more specific molecular and physical characteristics of tumours can be used to specify treatment modalities is obvious [9, 10]. Anatomical measurements are only one of many methods for stratifying breast cancer to understand the phenotypes of a particular patient better.

One of the most widely used tools for the classification of different subsets of solid tumours is the grading of histologically stained tumour sections. By analyzing the morphological appearance of tumours, the degree of malignancy is estimated and a course of treatment chosen [11]. Around the world, versions of the Nottingham System or Bloom Richardson Elston grading (BRE), so derived from the Bloom & Richardson method, are still used today to evaluate the aggressiveness of breast tumours by scoring neoplasia on three criteria: tubule formation, nuclear pleomorphism, and mitotic counts. Based on the abundance of each of these abnormalities, a tumour is scored and graded
[12]. These grades represent unique prognostic cohorts wherein patients with low-grade tumours have higher rates of disease-free survival and overall survival [12].

The derivation of new and often more sophisticated techniques for the identification of aggressive cancers is continually being pursued. In breast cancer, many of these techniques have centred around the loss of cell identity or dedifferentiation of cells, which has repeatedly been correlated with diminished outcomes for patients [13-17].

Clinical trials using dedifferentiation to categorize patients into treatment groups demonstrate that more differentiated lower-grade tumours respond better to therapies, and high-grade dedifferentiated tumours prove much harder to treat [12-14]. Proteins required for the normal function of the original tissue are concurrently lost as cancer develops and tumour cells lose their differentiated status. Two standard markers lost in the dedifferentiation of breast cancer are the sex steroid receptors (SSR) for estrogen (ER) and progesterone (PR) [18]. In studying the predictive value of SSR loss for patients receiving hormone therapy, not only were ER+/PR+ cancers more responsive to adjuvant hormone therapy, but they also have “independent predictive value in multivariate survival analysis [18].” Many of the improvements in women’s survival can be attributed to the advent of sex steroid receptor guided treatments [19]. The expression of a third protein, Her2, is used prognostically in conjunction with ER/PR status. The acquisition of Her2 expression typically results from gene amplification. This gene duplication event can project reduced time to relapse and decreased overall survival [20, 21]. It is clear from years of clinical data that the loss of ER/PR represents a significant development in disease progression associated with a loss of cell identity, which creates a significant barrier to successful treatment.

Since the implementation of these types of systematic grading procedures, reevaluations of their efficacy have been undertaken [12, 22-24]. Research to enhance these tools to better target treatments towards patients’ specific needs is an essential arm of cancer research. As the literature expands, so too do diagnostic capabilities, and consequently the ability to make finer distinctions about treatment regimens which can improve patient outcomes [25]. Since the first molecular subtyping experiment created the model for
using multi-gene expression panels to classify cancers, many similar tests have been developed [26]. The Genomic Grade Index exemplifies the growing sophistication of these tests by using 97 different genes to identify high- and low-risk groups within standard staging and grading cohorts [27]. In recent years, the value of these types of biomarker tests has been recognized for their clinical utility [28, 29]. The recognition and classification of breast cancer heterogeneity have helped expand our understanding of the disease and facilitated more directed therapies to the betterment of patients.

Genetic profiling and hierarchical clustering of microarray data were used to define ‘intrinsic’ molecular subtypes, which provide unique insights into cell states along the differentiation hierarchy, helping frame dedifferentiation and procurement of pluripotency. From these experiments, the five subtypes defined by gene expression are normal-like, luminal-like (with subcategories luminal A and luminal B), basal-like, HER2-enriched, and Claudin-low [26, 30, 31]. These molecular subtypes are characterized by their relatedness to normal cells. The normal-like cancers showed expression patterns typical of basal epithelial cells and adipose cells. Luminal and basal-like sub-types were likewise characterized by genes associated with their respective parental cells. The final group, from the initial study, was HER2 (ERBB2) positive, which often lost the expression of ER similar to the loss seen in the basal group.

Further research helped to identify the fifth ‘intrinsic’ group, Claudin-low [32-35]. The typical Claudin-low tumour is triple negative (TNBC — ER-/PR-/HER2-), but triple negative tumours represent a mix of all five molecular subtypes. These groups represent different patients with diverse clinico-pathological outcomes and unique molecular characteristics [33]. These ‘intrinsic’ subtypes show some overlap with traditional histological grading.

Claudin-low tumours have two notably elevated genomic signatures, epithelial to mesenchymal transition (EMT) and the tumour initiating cell signature or stem cell signature that helps distinguish them from the other subtypes [33]. These signatures correlate with a more aggressive phenotype and were most up-regulated in Claudin-low tumours [33, 36-40]. Using a model constructed from microarray data gathered during the differentiation of mammary stem cells to luminal progenitors and finally into mature
luminal cells, a differentiation continuum was built. Breast cancer molecular subtypes segregated along this continuum [41] with luminal subtypes being the most differentiated, followed by HER2-enriched tumours, then basal-like, and finally undifferentiated Claudin-low tumours [33]. Using immunofluorescence all subtypes were positive for cells of epithelial origin that were also expressing mesenchymal markers, but the most dedifferentiated tumours, Claudin-low and basal-like, had more frequent mesenchymal features suggesting some degree of EMT was occurring in all of the tumours [33]. Differentiation status as it relates to EMT and stemness is a vital contributor to the intertumoural and intratumoural heterogeneity that makes breast cancer challenging to treat [42].

Though inter-patient differences create enormous clinical difficulties, the heterogeneity of an individual’s tumour is key to understanding tumour development, mechanisms of chemoresistance, and recurrence. As early-stage lesions grow and become increasingly malignant so too does the diversity of gene expression in the neoplasia. In most grade II (Nottingham) premetastatic lesions isolated by laser capture microdissection, a mix of two gene signatures were identified. The gene signatures were those of grade I and the grade III cancers indicating a heterogeneous mix of cells very early in the transformation process [43]. An extensive study of ductal carcinoma in situ (DCIS) microarray analysis confirmed the presence of luminal, basal, and HER2+ subtypes that comprise 80% of the lesions tested. Nearly half of all DCIS were found to have areas consisting of multiple nuclear grades, and approximately 9% of the pure DCIS displayed all three nuclear grades. In many cases, individual DCIS tumours contained multiple subtypes. These lesions represented nearly every combination of intrinsic subtype [44]. These data were obtained using histological biomarkers (ER, GATA3, ERBB2, CK5/6, CK18, and p53) as surrogates for subtypes [44]. It is clear that heterogeneity, and the adaptation it confers, are problems arising in the earliest stages of malignant transformation and affect the entire natural history of a patient’s cancer.

1.1.2 Origins of Intratumoural Heterogeneity

Many sources of intratumoural heterogeneity — the phenotypic variability amongst cells of the same tumour originating from genetic, epigenetic, and environmental differences
— have been studied. From these studies, there has emerged two prevailing theories, the cancer stem cell hypothesis, and the clonal evolution model [45, 46]. The cancer stem cell theory states that a small subset of cancer stem cells (CSCs) capable of initiating new tumour growth is also responsible for producing the heterogeneous progeny that populate a tumour. This concept was postulated as early as the 1870s, though it was not systematically studied as a theory until the late 1990s [47, 48]. Early data supporting this idea came from observations made regarding the rates of colony formation in *in vitro* assays, and the rate of tumour formation from limiting dilution tumour implantation assays in mice [49-51].

The next major advancement in the cancer-stem-cell hypothesis was the identification of cell surface markers (CD34+CD38−) that could be used to identify a sub-population of acute myeloid leukemia (AML) cells that when transplanted into NOD/SCID mice formed tumours at a higher rate than the rest of the population. Fluorescence activated cell sorting (FACS) and xenotransplantation experiments like this have been instrumental in understanding the self-renewal capacity of CSCs. Since this discovery, cells exhibiting enhanced plasticity and self-renewal have been identified in many solid tumours. Starting with the discovery of these cells in breast cancer the list has now grown to include glioblastoma, melanoma, osteosarcoma, chondrosarcoma, prostate cancer, ovarian cancer, gastric cancer, and lung cancer [52-60]. It is notable that conflicting data regarding the frequency and hierarchy of CSCs exist for melanoma [61]. In melanoma, if given the correct conditions, cells have been demonstrated to produce tumours from a single primary cell [61]. In reconciling the differences in the behaviour of melanoma and other cancers we may be able to derive significant insights into the nature of plasticity in cancer. A model that is used to explain these differences, termed phenotype switching, was described by Hoek & Goding in 2010, who outlined the idea that plasticity is acquired, lost and re-acquired throughout melanoma progression. This idea contrasts with the notion that CSCs asymmetrically and terminally differentiate into highly proliferative cancer cells. The ability to adapt to environmental cues by transiently expressing stemness is referred to as phenotypic plasticity. This concept was experimentally demonstrated in melanoma by Roesch et al. in 2010 [62]. Within melanoma tumours, there exists a population of slow dividing JARID1 (jumonji/ARID1 histone 3 lysine 4
(H3K4) demethylases) positive cells that give rise to the rapidly dividing progeny and are necessary for the continued propagation of a tumour. These cells are shown to represent a temporary state, which can be acquired by JARID1 low cells. Whether derived from hierarchical differentiation, acquired dynamically, or a combination of both, CSCs and plasticity are a significant contributor to tumourigenicity and thus crucial factors in the maintenance and treatment of disease.

The opposing clonal evolution model postulates that cell populations accumulate hereditary changes that confer a selective evolutionary advantage, which allows those changes to be inherited more frequently [63]. Deep sequencing and single cell sequencing have largely confirmed the existence of clonal evolution [64, 65]. Using these data to reconstruct genealogical trees marks a significant advancement in our tools for discovering the order in which mutations are acquired, so we may better understand the process of tumour evolution. It is likely that when we marry these two models, cancer stem cell and clonal evolution, that we approach a more accurate description of the truth. In *The Origins and Implications of Intratumor Heterogeneity*, two different hypotheses are offered to explain how both theories could co-exist [66]. The first is that plastic cells that persist in a tumour produce transit-amplifying cells, which are persistent and capable of differentiation. If the transit-amplifying cells persist long enough to acquire heritable changes that confer enhanced plasticity, they will survive and become part of the heterogeneous makeup of a tumour. The second proposal is that there exists a continuum of tumour plasticity. Throughout a tumour exist cells with varying degrees of plasticity, resulting from microenvironmental signals [66]. Regardless of the specific model by which cells acquire plasticity, its importance and the importance of tumour heterogeneity are broadly accepted.

1.1.3 Breast Cancer Stem Cells

Breast cancer stem cells are best defined by two characteristics: self-renewal and plasticity [67]. The gold standard for testing these traits is serial transplantation in animal models. Only in performing the second transplantation do we see if the cell that developed into the first tumour retained its capacity for self-renewal. Due to the cost and time-consuming nature of these experiments, surrogates are often used such as sphere
formation assays, colony forming assays, or gene expression [68, 69]. From these types of experiments a model of asymmetrically dividing cancers cells capable of enhanced neoplastic traits emerges.

Cell sorting using cell surface markers has become the cornerstone of CSC experiments. In breast cancer, CD24 and CD44 adhesion molecules were chosen as prospective candidates to isolate a population of cells better capable of initiating tumours [52]. Since the original experiment in 2003, many labs have verified CD44^{high}/CD24^{low} as a reliable means of detecting breast cancer cells with increased stem cell characteristics [70-72]. Because these markers are surrogates correlated with much broader underlying phenotypes, refinement of the techniques to better identify CSCs are consistently under investigation. CD44 does have a direct role in supporting plasticity, though it is still not sufficient for the identification of all CSCs. In the Al-Hajj study, only 8 of 9 tumours expressed CD44 [52]. ALDH1, CD133, CD44+ CD49fhi CD133/2hi+ have been used to identify stem-like populations in breast cancer [36, 73-75]. These alternate markers and attempts to further refine CSC isolation provide more precise tools to examine the molecular and phenotypic characteristics of CSCs.

Because we must use cell surface markers as stand-ins for phenotypes combined with the inability to test serial transplantations in immunocompetent autologous orthotopic environments, we cannot know the actual frequency of CSCs. The most extreme example of this is found in melanoma where CSC rates in xenotransplantation experiments range from 0.1% to 0.0001% [61]. Our understanding of the mechanisms by which CSC frequency is maintained in breast cancer is relatively rudimentary, but the rate of asymmetric division and stem cell dormancy have been shown to regulate the process in breast cancer [76, 77]. As long as a model is sensitive enough to detect changes in plasticity and self-renewal, those changes and what drives them may be more valuable than absolute CSC frequency.

Two of the chief claims concerning CSCs are their importance in metastasis and their role in resistance to therapy. These are of particular concern because metastatic breast cancer causes the vast majority of breast cancer-related deaths. There is only a 26% five-year
survival rate for metastatic breast cancer [78, 79]. Annual recurrence rates for the first five years are about 10% [78, 79]. These recurrent tumours are more difficult to treat and more likely to be fatal. Both indirect and direct evidence suggests that CSCs play a critical role in tumour dissemination and metastasis. Several studies show this in different ways: the presence of CSCs in primary tumours correlates with metastatic spread; CSCs possess a dedifferentiated invasive gene signature that correlates with patient metastasis; and ALDH1 activity is also associated with the prevalence of tumours at secondary sites [68, 80, 81]. As more direct evidence; a subset of CD44+ CSCs was shown enhanced spontaneous metastasis to the lungs; early disseminated bone metastases showed CSC phenotypes; and circulating tumour cells (CTCs) displayed enhanced stem cell phenotypes [82-84]. The ability of the plastic phenotype, EMT, to caused therapy resistance has been shown in breast cancer [85, 86]. If plasticity confers resistance to therapy, it will follow that CSCs should be resistant to therapy as well. Many current chemo- and radio-therapies are most effective on highly proliferative cells [87-91]. Breast CSCs can reduce their rate of proliferation entering a quiescent or dormant state reducing the efficacy of these types of treatments [87-91]. The low reactive oxygen species (ROS) levels found in CSCs are protective against radiotherapy compared to the non-tumourigenic cancer cell population due in part to increased expression of free radical scavenging systems [92]. Patient data from HER2+ and HER2- breast cancers show an intrinsic resistance of breast CSCs [93]. CSCs from breast cancer cell lines have higher levels of anti-apoptosis signals and express higher levels of ABCG2, a protein that contributes to multi-drug-resistant breast cancer [94-96]. CSC-driven metastasis and therapy resistance derives from multiple pathways that support these malignant phenotypes. Deriving new therapies that counteract these phenomena are integral to decreasing adaptations that lead to therapy resistance.

One complication in deriving therapies that specifically target CSCs or their defining characteristics of self-renewal and plasticity, is that CSCs may not be a small, indelible subset of cancer cells, but an acquired state characterized by the acquisition of the aforementioned phenotypes. In the SUM159 and SUM149 breast cancer cell lines when cell were FACS sorted into three groups, stem-like, basal-like, and luminal-like, the growth of any one of the sorted populations resulted in the rapid repopulation of the
removed subtypes much faster than could arise from cell division alone, suggesting trans-differentiation is occurring [97]. The interconversion of non-CSCs to CSCs can be facilitated by IL6 or can happen naturally in tumours formed from non-CSCs given enough time. Though these cancer cells could convert bidirectionally, the process of creating non-CSCs from CSCs was less efficient in the absence of stimuli [98]. These data are confirmed by the sequencing of CSCs and non-CSCs, which contain few, if any, genetic differences, indicating the likely interconversion of CSCs and non-CSCs. If CSCs are in part a state and not a permanent subtype of cells, we could expect to see a heterogeneous expression of pluripotency and self-renewal genes [99]. Expression of dnMAML in MCF7 cells, helps identify one factor in this type of heterogeneity. In MCF7 cells dnMAML blocks all Notch signalling, reduces but does not abate tumour formation from CSCs thereby elucidating the presence of a Notch-dependent and a Notch-independent subset of CSCs [100]. The isolation of CSCs provides a powerful tool for understanding intratumoural heterogeneity by making it easier to study the cells expressing the phenotypes of interest. An integral part of this research will be identifying and quantifying the limitations of state-dependent and lineage-acquired plasticity.

1.1.4 Acquiring Phenotypic Plasticity to Drive Tumour Heterogeneity

How developing neoplasia acquire a stem cell state and the dynamics of said state throughout tumour development are necessary areas of study. Understanding how tumours change and adapt by diversifying cellular phenotypes is essential for rational therapy design. For example, if dedifferentiation is primarily unidirectional, this poses a different technical challenge than if cancer cells can transition out of a multipotent state in response to a drug targeting that phenotype, especially if the residual tumour cells can reacquire plasticity once treatment is complete. Numerous studies have described the acquisition of pluripotency, or an increase in CSC abundance in response to different factors and will be discussed below. From these data, a single unified theory for the acquisition of plasticity as a phenotype has yet to arise. It is likely that many factors contribute to the attainment of a progressively more plastic cancer. We may be best served by schematizing cancer as having progressive stepwise development with concurrent evolving populations. As tumourigenesis progresses plasticity is increased in
general, but the diverse set of populations produced by plasticity then occupy their own niches and begin their own parallel but divergent evolutions.

1.1.4.1 Mutations as Drivers of Plasticity

Mutations affect numerous cellular processes like proliferation, apoptosis, and DNA repair, among many others, leading to and exacerbating neoplastic transformation and disease progression. New evidence is accumulating that mutations can also drive a multipotent phenotype. Evidence from several studies of BRCA1/2 mutations suggests that the loss of function of either gene can cause stemness to increase [72]. Corollary evidence is found in patient tissue sections in which the loss of BRCA1 correlates with CD44 positive cells [101]. Using an siRNA knockdown strategy in breast cancer cell lines to approximate BRCA1 deficiency, Buckley et al. demonstrated the BRCA1-loss mediated decreased levels of ERα and luminal marker expression. The dedifferentiation of these tumours resulted in an increase in tumour sphere formation with accompanying elevated ALDH activity, and an expansion of the stem cell-like population in flow cytometry experiments [102]. The plastic phenotype observed was determined to be regulated through Notch1 and JAG1, and alterations in these pathways were verified in BRCA1 deficient patient samples [102]. Maybe the most compelling data that suggest that mutations affect plasticity is in a study of parous, nulliparous women, and women harbouring BRAC1/2 mutations [103]. Healthy breast tissue was tested for CD44 expression, the positive cells were isolated, and their gene expression was analyzed. Unsurprisingly, the most perturbed pathways in BRCA1 mutation-bearing women were found in DNA repair pathways [103]. The highest scoring pathways in women carrying BRCA2 mutation were those involved in stem cell function, development, and differentiation [103]. It is reasonable to hypothesize that one means of achieving an eventual multipotent state is either through germline mutations or mutations obtained early in transformation that alter the expression patterns of pathways regulating cell fate.

1.1.4.2 Plastic Phenotype EMT Drives Carcinoma Progression

Conceptually there are several observations that relate the loss of cell fate to the differentiation status of tumours. First is the histological dedifferentiation of tumours,
which present as structural or morphological changes. These are often reflected in grading systems in the form of tubular formation, nuclear-to-cytoplasmic ratio, or loss of polarization [104]. Second are the changes in gene expression such as the loss of tissue-specific differentiation markers, but also includes the reemergence of previously silenced stem cell genes, which may grant cells the characteristic phenotypes of stem cells [14, 26, 103, 105, 106]. The third and maybe most thoroughly researched is epithelial to mesenchymal transition. EMT is a multifaceted process by which epithelial cells acquire mesenchymal properties. This process occurs naturally in development and wound healing, and aberrantly in tumour progression [107-110].

During malignant progression, EMT governs many core tumourigenic developments including loss of polarization, adhesion, migration, vascular survival, extravasation and metastasis [111-116]. EMT is a naturally occurring plastic phenotype that, like other aspects of phenotypic plasticity, is co-opted and deregulated in cancer. EMT’s plastic phenotype can be induced by cell-extrinsic factors like extracellular TGFβ, hypoxia, or tumour-stromal interactions [117-120]. Regardless of what signal acts as the inciting incident initiating EMT, the signals converge on a set of potent transcription factors, Snail, Slug, Twist and zinc-figure E-box-binding homeobox (ZEB1 and ZEB2), that are classified as EMT-inducers [113, 121-124]. These transcription factors are found to be dysregulated in breast cancer and more specifically upregulated in metastatic disease [125]. EMT-inducing transcription factors Twist and Snail are commonly upregulated in circulating tumour cells of non-metastatic breast cancers and found in 100% of the patients with metastatic breast cancer in these studies. These two factors also demonstrate independent prognostic value and correlation with stage in late-stage disease [125-127]. EMT is a phenotype that is important at multiple points in tumourigenesis. Early in disease progression, it has been shown to be important in the transition from low-grade DCIS to high-grade DCIS, and it is also a notable characteristic of basal-like, triple negative, and Claudin-low tumours [33, 128-130]. EMT is a process by which cells dedifferentiate, acquiring traits from cells of the mesenchymal lineage. This plasticity produces cells that are more motile and invasive thus better capable of metastasizing.
Emerging data shows EMT not only to be a plastic phenotype utilized by cancer but also to increase other stem cell-like properties. TGFβ, Snail, and Twist confer stem cell-like phenotypes in immortalized human mammary epithelial cells including the emergence of a CD44\textsuperscript{high}/CD24\textsuperscript{low} population that increased spheroid formation [131]. TGFβ also increased EMT and CD44\textsuperscript{high}/CD24\textsuperscript{low} cells simultaneously which lead to increased tumour outgrowth in xenotransplantation assays [117].

To truly appreciate the plasticity of cells that have undergone EMT, we can look at this feature throughout metastasis. A study of this process in MDA-MB-468 cells show that the primary tumour exists in a hybrid state of partial EMT expressing both E-cadherin and Vimentin. As the tumours develop and invade cells lose the expression of E-cadherin advancing towards the mesenchymal lineage, but lymphovascular emboli re-expressed E-cadherin indicating they had undergone a reversal of EMT termed mesenchymal to epithelial transition (MET) [132]. This concept is well supported in the literature [132-134]. Fluid transitioning between cell states provides a powerful means of adaptation and with EMT’s ability to also confer stemness, these changes in differentiation are even more extensive than just the acquisition of mesenchymal features.

1.1.4.3 The Tumour Microenvironment Supports and Enhances Phenotypic Plasticity

The tumour microenvironment is a broad term that encompasses cell extrinsic aspects that affect tumours. Extracellular matrix (ECM), paracrine factors, hypoxia (which will be discussed in detail later), pH, and resident non-transformed cells like fibroblast and immune cells all constitute features of the microenvironment that contribute to cancer progression [135-144].

Insights from parallel work in the iPSC and stem cell fields may inform our understanding of how the ECM can regulate plasticity and differentiation. This work demonstrates that ECM substrates or 3D culture can be used to regulate the plasticity and differentiation of iPSCs and ESCs [145-148]. The effect of the ECM can be so profound that it is capable of partial re-differentiation of cancer cells. Melanoma cells that were grown on ECM conditioned by, then stripped of hESC, caused reprogramming of the
cells resulting in the re-expression of Melan-A and decreased invasiveness [149, 150].

The re-differentiation caused by hESC conditioned ECM reintroduces factors responsible for regulating signals from the ECM that are lost in cancer. hESCs-conditioned-ECM is also capable of decreasing anchorage-independent growth, tumour volume, and proliferation of breast cancer cells [151]. The Nodal antagonist Lefty was identified as a key regulatory factor that is deposited by hESCs into the matrix. Lefty remains silenced in cancer even as Nodal expression re-emerges. Reintroduction of hESC-derived Lefty decreases Nodal expression and colony formation in aggressive breast cancer cell lines [151]. Nodal and Lefty may both utilize the ECM for extracellular signal transduction suggesting a central role of the ECM in facilitating paracrine signalling [152, 153]. The ECM may then have the dual roles of supporting pluripotency through facilitating paracrine signalling and through direct interactions.

Hyaluronan synthase 2 (HAS2) is an enzyme that remodels the ECM by producing hyaluronan that is expressed more highly in a metastatic subpopulation of CSCs leading to the greater outgrowth of bone metastases [154]. One mechanism for this increase in tumourigenicity is that tumour-HAS2 increases hyaluronan in the microenvironment, which binds CD44 increasing EMT and stemness via ZEB 1 and CD44 splicing [155, 156]. Not only can the composition of the ECM alter cell state, but even increased stiffness can support plasticity by inducing TAZ signalling, which then promotes self-renewal and tumourigenic potential of breast cancer cells via induction of CSCs [157, 158].

Compelling evidence regarding the role of periostin (POSTN) in the acquisition of plasticity has been accumulating for years. POSTN can expand CSC populations expressing high levels of CD44 and low levels of CD24, increase resistance to chemotherapy, promote stemness as measured by sphere formation, and increase mesenchymal traits via EMT [159, 160]. POSTN is one of many possible links between ECM and non-tumour cells recruited to aid tumour growth. Both fibroblasts and immune cells in the lung stroma can be induced to express POSTN by infiltrating cancer cells [159, 160]. This induction of expression supports metastasis by restructuring the
secondary sites in the lung to better support tumour growth. Tumour cell-derived TGFβ3 mediates the upregulation of POSTN in resident fibroblasts [159, 160].

1.1.4.4 Epigenetics

One of the newest and most intriguing areas of study regarding plasticity in cancer is the role of epigenetic regulation. Epigenetics was functionally defined as “a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence [161].” These heritable phenotypes are controlled by several mechanisms which include but are not limited to: DNA methylation, histone modifications, histone variants, and nucleosome arrangement. Chromatin regulation is a required part of differentiation and lineage specification (reviewed in [162]).

Naturally occurring differentiation requires large-scale chromatin rearrangement affecting nearly one-third of chromatin structure [163]. These alterations consisted of changes in histone methylation, with specific expansion of H3K9me3 and H3K27me3 domains. H3K4me3/H3K27me3 bivalent promoters were 32% more common in hESCs compared to fibroblasts, a trait discovered in ESCs to allow chromatin to respond rapidly to developmental cues [104]. It is not only global changes in epigenetic modifications that can be observed, but pluripotent cells also exhibit chromatin structure that is more dynamic, continually undergoing nucleosome exchange and remodelling [164, 165]. Expansion of methylation at H3K9me3 and H3K27me3 domains revealed a pattern of enrichment in genes associated with developmental processes and lineage specification indicating a concerted regulatory role, which governs cell fate [53, 164]. Cessation of pluripotency is regulated by unique combinations of repressive marks [164, 165]. With the revelation that somatic cells can be made to reacquire pluripotency through reprogramming, Hawkins et al. compared fibroblast to fibroblast-derived iPSCs and observed a return in the global epigenetic landscape to one comparable to hESCs, though several incorrect marks still exist suggesting some residual effects of differentiation [163]. Using stem cells, iPSC and naturally occurring differentiation provide a base understanding of the relationships between pluripotency and epigenetics.
It would follow that the reacquisition of plasticity in cancer would require alterations in epigenetic regulation facilitating either the re-expression of tumour-supporting plasticity genes or the silencing of lineage-specifying genes. Mutations in various epigenetic regulators have been reported in breast cancer like ARID1B, ARID2, SMARCD1, ASXL2, ARID5B, KDM3A, SETD1A, CHD1, CHD5, MBD1, MBD4, NCOR1, NCOR2, HDAC9 and CTCF [166-168]. One-third of pediatric glioblastomas harbour histone H3 mutations. The most common being K27M substitution in H3F3A gene, which reduces repressive histone H3K27me3 mark reverting cells to a neural precursor state and imparting ‘oncogenic self-renewal’ [169, 170].

DNA hypermethylation commonly associated with transcriptional silencing is found in the promoters of tumour suppressor genes in multiple cancers including breast [171, 172]. This silencing was found to be more extensive in more aggressive cancer cell lines [171]. Expectedly, the converse is also true as decreases in promoter methylation and changes in histone marks upregulate NANO2, OCT4, SOX2 and c-MYC gene expression. [173, 174]. Rivenbark et al. also demonstrate that these changes can be reversed in a targeted manner. Cells were treated using zinc-finger (ZF)-based artificial transcription factors (ATFs) with methyltransferase activity to directly methylate the SOX2 promoter, which led to promoter repression and downregulation of SOX2 [174]. This methodology may provide a method for future experimentation or targeted epigenetic reprogramming. In mice, breast tumours expressing high levels of Nanog (>1.8 fold) had promoters associated with the bivalent histone marks — H3K4me3 and H3K27me3, which is notably associated with a dedifferentiated state in stem cells [175, 176]. Genome-wide analysis shows similar methylation patterns between less aggressive luminal cancers and CD24high non-CSCs, and more aggressive tumours (HER2+ and basal-like) and CD44high CSCs. DNA hypomethylation in the latter group affects broad sets of genes including those regulating plasticity [177, 178].

Broader changes in chromatin structure also help support plasticity in multiple cancers including breast. Histone H1.0 expression is highly heterogeneous in breast cancer [179]. Cells bearing CSC markers have low levels of H1.0, and differentiated tumour cells possess higher levels of H1.0. Histological grade inversely correlates with H1.0. The
absence of H1.0 supports plasticity by destabilizing the interaction between DNA and nucleosomes to coordinate de-repression of large gene sets. Knockdown of H1.0 increases tumour growth and aggressiveness [179].

1.1.4.5 EMT and Epigenetics

EMT plays a direct and active role in regulating the epigenetic landscape. In breast cancer, Twist directly interacts with SET8 methyltransferase to regulate EMT via its H4K20 monomethylation activity [180]. Snail has also been shown to direct epigenetic changes promoting EMT via recruitment of the Sin3A/histone deacetylase 1 (HDAC1)/HDAC2 complex to the E-cadherin promoter [181]. Snail has another binding partner that it recruits to epithelial gene promoters to silence them, histone demethylase LSD1 (KDM1A) [182]. LSD1 induces CSCs and plastic phenotypes in breast cancer and is responsible for maintaining accessible chromatin structure for genes like POU5F1 (OCT4) and NANO [183, 184]. In basal, but not luminal, breast cancer cells changes in ZEB1 expression can interconvert CD44\textsuperscript{low} cells to CD44\textsuperscript{high} CSC-like cells as measured by flow cytometry, sphere formation, and tumour growth in xenografts [120]. To determine if the differences in regulation were facilitated by epigenetic marks found at the ZEB1 promoter of luminal, CD44\textsuperscript{low} basal, and CD44\textsuperscript{high} basal, breast cancer cells were analyzed. The luminal promoter possessed only inhibitory marks. The CD44\textsuperscript{high} basal cells were relatively high in H3K4me3 and H3K79me2 with infrequent H3K27me3, indicating active transcription. The CD44\textsuperscript{low} basal cells were the most exciting group — with both H3K4me3 and H3K27me3 present they exist in a bivalent state ‘poised for activation.’ These diverse initial states may explain the differential effects of ZEB1 on luminal and basal tumours [120]. Interconversion of CD44\textsuperscript{low} basal, and CD44\textsuperscript{high} basal breast cancer cells can be induced by exposure to the common microenvironmental factor and regulator of EMT, TGFβ, which in this model increases the accessibility of the ZEB1 promoter by reducing the H3K27me3 repressive mark [120]. The study of EMT provides the best evidence that plasticity and epigenetics are far more dynamic processes than previously thought. The constant writing and rewriting of the epigenome facilitates the highly dynamic adaptation, differentiation, and de-differentiation that underlies intratumoural heterogeneity.
1.1.4.6 Stem Cell Signature

However the seeds of pluripotency are obtained, the existence of the phenotype necessitates an alteration in gene expression [185, 186]. In breast cancer tumour grade, histological differentiation and size all correlated with a global increase is ES gene signatures, with these signatures being critical features of high-grade breast cancer tumours [105]. As Ben-Porath, et al. clearly articulate, these types of studies are limited by our understanding of the process by which ES cells differentiate into specific tissues. This information would allow tailoring of gene signatures to the particular tissue from which a tumour arises [105]. Three transcription factors were consistently upregulated when comparing breast cancer to ECSs: NANOG, OCT4, and SOX2 [105]. Beyond these core factors, a broader subgroup of transcriptional regulators was found to be highly expressed in ESCs and high-grade tumours. The importance of these central pluripotency factors in phenotypic plasticity of certain cancers has repeatedly been observed either as a ‘core pluripotency’ signature or as individual genes promoting tumour progression [106, 175, 187, 188]. These types of embryonic or stem cell genes have garnered interest for their ability to enhance tumourigenesis and intensify metastatic phenotypes [105, 115, 175, 189].

KLF4, a factor used in the initial creation of induced pluripotent stem cells (iPSCs) is upregulated in ~70% of DCIS breast tumours [190, 191]. Fang Yu et al. demonstrate that KLF4 increased CSC populations in primary mouse models and human breast cancer cell lines, which correlated with heightened ALDH1 activity. The KLF4-mediated maintenance of the stem cell population was regulated independently of its effects on migration and invasion, which were Notch1 dependent. Aberrant expression of stem cell factors that help develop and maintain the pluripotent phenotype are imperative to our understanding of the etiology of cancer. Identifying how these pathways differ from their correctly regulated counterparts in stem cells will be instrumental in characterizing the pathways. It may prove to be that the vital research question is not ‘how are stem cells and cancer similar?’ but ‘how are they different?’

A good illustration of the complexity of these systems is the relationship between EMT, cancer stem cells and the stem cell signature. As previously discussed, EMT promotes
stemness, but the converse is also true [131, 192]. OCT4 and NANOG also regulate EMT by increasing the expression of N-CADHERIN, VIMENTIN, SLUG, and SNAIL in CSCs [192]. When OCT4 and NANOG were knocked-down, EMT gene expression could not be recovered by TGFβ, but TGFβ did increase E-CADHERIN and CK-18. Further confounding these data, TGFβ activated SMAD3, a signal transducing protein that binds OCT4 and NANOG promoters in other processes, so it is possible given the correct microenvironmental and epigenetic context that TGFβ could upregulate OCT4 and NANOG [193, 194]. When taken together in this way, it is clear there is a complex non-linear network with probable redundancy driving stemness and tumour heterogeneity. Intratumoural heterogeneity itself creates a broad range of cellular phenotypes that respond differently to treatments, so without finding a shared characteristic of pluripotent cells, be it a microenvironmental cue, a linchpin master regulator, or a shared feature of pluripotency genes, broadly functioning and reliable treatments will remain elusive. The other possible path forward is specific treatments for more refined subtypes of breast cancer defined in part by plasticity.

1.2 Hypoxia in Breast Cancer

The clinical/physiological definition of hypoxia is a state of reduced oxygen availability below the threshold necessary for normal function of the organ, tissue, or cell [195]. Solid tumours often contain areas in which the oxygen tension is far below that of the healthy resident tissue [196]. In the analysis of ten independent studies, normal breast tissue was found to have a median oxygen partial pressure (pO$_2$) of 65 mg Hg with no patients having a measurement less than 10 mg Hg. In contrast, the median pO$_2$ of breast tumours, as well as three other cancers, was 10 mg Hg. In many tumours, areas of more severe hypoxia were observed. Hypoxia has been detected in 50% of locally advanced breast tumours and has been correlated with poor clinical outcomes [197]. Hypoxia used in this way as a prognostic factor is independent of clinical tumour size, stage, grade, and histopathological type [198]. More recent evidence supports this correlation. By defining a hypoxic signature, researchers could stratify patients based on their hypoxia response and predict clinical outcomes [199]. A genetic test like this would be more readily adopted in a clinical setting than a physiological measurement of oxygen tension.
Hypoxia is a relatively common and clinically significant feature of the solid tumour microenvironment, which may be used to guide treatment and improve clinical outcomes [200].

1.2.1 HIF1α Mediated Response to Hypoxia

The master regulator of hypoxia signalling is HIF1α. In normoxia three prolyl hydroxylases (PHD 1, 2, and 3) are responsible for adding prolyl groups to the two von Hippel-Lindau binding domains [201-203]. This reaction is O₂-dependent allowing it to act as a sensor for molecular O₂. The prolyl groups facilitate binding of E3 ubiquitin ligase von Hippel-Lindau tumour suppressor protein (pVHL) to one or both of HIF1α’s two oxygen degradation domains leading to polyubiquitylation-mediated proteasomal degradation [201, 204-206]. In hypoxia unhydroxylated HIF1α translocates to the nucleus where with its binding partner HIF1β binds to enhancer regions termed hypoxia-responsive elements. The HIF1α/HIF1β complex then recruits transcriptional coactivators to regulate gene expression [207, 208]. Most results from the literature regarding HIF1α can be hypothesized to also be true for hypoxia, but these ideas should be tested as not all effects of low oxygen are HIF1α-mediated [209]. The other confounding factor is that HIF1α is not exclusively utilized to respond to low levels of molecular O₂. The HIF1α level can be regulated by other means; surprisingly this even includes reoxygenation [210, 211]. Other commonly used hypoxia mimic is CoCl₂ and DFO, which mimic the activation of many hypoxia pathways by disrupting heme moieties, but fails to activate other central hypoxia responses [212]. Though these tools are undoubtedly useful in providing insights into the effects of hypoxia on cancer cells these results should not be generalized without direct evidence to do so.

1.2.2 Hypoxia’s Role in Breast Cancer Progression and the Acquisition of Plasticity

Hypoxic stress arises from rapid tumour growth, resulting in a compromised and disorganized microvasculature that leads to adverse oxygen diffusion gradients [196, 213]. Lack of blood supply creates coincident stresses such as energy and nutrient depletion and accumulation of harmful metabolic byproducts [196]. When oxygen
tension is low enough and sustained for long enough, it can induce apoptosis-mediated cell death, which constitutes a lack of cellular function adhering to the physiological description of hypoxia [195, 214]. The most straightforward mechanism by which a harsh apoptosis-inducing microenvironment can promote neoplastic progression is through clonal selection, an observation which has been experimentally verified by many groups [215-217]. Further supporting this evolutionary selection theory is a substantial body of work indicating that increased genomic instability results from exposure to hypoxia [218-220]. The ability to produce a genetically diverse population of tumour cells and then apply the selective pressure required to enrich for more tumourigenic lineages is an ideal milieu to facilitate tumourigenesis.

Hypoxia acts as a selective pressure causing cell death in some cells, but the surviving population alters their gene expression and the subsequently regulated phenotypes to promote neoplastic progression [215, 217]. These changes often occur in pathways regulating survival, proliferation, energy homeostasis, angiogenesis, invasion, migration and metastasis. In colorectal cancer, 24h exposure to hypoxia increases resistance to apoptosis and promotes proliferation. These phenotypes are maintained upon reoxygenation [215, 221]. These changes are often facilitated by the upregulation of anti-apoptotic proteins like Survivin. Hypoxia increases the expression of Survivin in breast cancer in a HIF1α dependent manner, and its expression is linked to the prevention of apoptosis [222-224]. It is worth noting that Survivin is not only an anti-apoptotic protein but one that is expressed primarily in embryonic and cancerous tissues — not in healthy adult tissues [225]. Cells surviving their exposure to hypoxia and maintaining the alterations imposed by the stress may acquire progressively more advanced neoplastic phenotypes.

Hypoxia activates multiple survival mechanisms in breast cancer. Autophagy is employed in hypoxia as a means of recycling proteins and clearing damaged organelles, which aids in survival [226-228]. Lack of oxygen drastically alters glucose metabolism shifting function away from the electron transport chain to glycolysis, an adaptation often required for survival. A side effect of this process is extensive lactic acid production that modifies the tumour microenvironment promoting transformation [229-231]. In hypoxia
energy is scarce and cancer cells often reduce the total level of translation to conserve energy while utilizing alternate forms of translation initiation to selectively express those genes necessary for survival [232-235]. Adaptation to hypoxia dramatically alters cellular physiology. A subset of these changes is maintained after reoxygenation [236]. Repetition of this cycle will lead to gradually more tumourigenic and harder to treat cancer cells.

The accumulation of pro-tumourigenic phenotypes in response to hypoxia affects nearly all aspects of tumour progression. Primary tumour growth in breast cancer is regulated in part by CA9, a protein robustly upregulated by hypoxia [237]. Inhibition of CA9 reduces tumour growth [237]. This work parallels the results seen in HIF1α-null mouse breast tumour cells, which showed significant retardation of tumour growth compared to wild-type cells [238]. Access to the circulatory system is a vital stage in metastasis. HIF1α and hypoxia cause angiogenesis, new blood vessel growth from existing blood vessels, either by secreting factors or by stimulating non-tumour cells to promote angiogenesis [239-242]. The formation of new blood vessels ultimately provides oxygen and nutrients that support bulk tumour growth [243]. Accordingly, microvascular density correlates with metastasis in breast cancer [244]. A thoughtful example of the signalling complexity regulating angiogenesis can be seen in the work of Braunstein et al. where they demonstrated that hypoxia causes the expression of VEGF, leading to increased tumour vascularization and tumour size. These changes resulted from global alterations in translation mediated by the activation of 4E-BP1 [245]. Invasion, the process by which tumour cells penetrate surrounding tissues, is also regulated by hypoxia. The mechanisms for hypoxia-driven invasion in breast cancer include the induction of lysyl oxidase (LOX) and the initiation of EMT [246-249]. The latter can be accomplished through the activation of numerous pathways [247-249]. As hypoxia causes tumours to grow, invade, and gain access to the vascular system, it follows that it would also facilitate metastasis. In MMTV-Neu transgenic mice hypoxia increases macrometastases and macrophage tumour infiltration [250]. Intermediate hypoxia (24h) caused an increase in metastases to the lung. This exposure also increased some markers of EMT: genes associated with glucose metabolism; a set of genes previously shown to predict lung metastasis; protumourigenic cytokines; and a panel of stem cell markers [250]. The intermediate
exposure to hypoxia increased tumour initiating cells as measured by cell sorting and limiting dilution transplantation assays [250]. The ability for hypoxia to support CSCs and plastic phenotypes in breast cancer has been replicated many times. These changes seem to result from extensive signalling involving miRNA, cell surface markers, recruitment of non-transformed cells, stress responses, and metabolic changes [211, 250-254]. Hypoxia arises early in tumour development and exerts pressure contributing to a broad range of cancer phenotypes. To date, it has proven difficult to turn these data into actionable clinical practices.

1.2.3 Hypoxic Stress Promotes Therapy Resistance

Hypoxia not only contributes to oncogenesis, but it also alters the tumour in ways that make many conventional therapies less effective [255, 256]. Many have observed that hypoxia limits the effectiveness of radiotherapy. The earliest known mechanism for radio-resistance was that reduced oxygen stoichiometrically limited the creation of adducts — difficult to repair single-strand DNA breaks [255, 256]. Other mechanisms are less directly associated with molecular oxygen and result from cellular adaptation to stress. Maintenance of energy homeostasis through reducing protein synthesis or activation of HIF1α regulated pathways and stress granule formation, coordinate to produce an adaptive protein response that is radio-protective [210, 257, 258].

Like with radio-resistance, hypoxia also increases chemoresistance [259]. This resistance results from a combination of structural, chemical, and biological factors that each contribute to hypoxic tumours being less responsive to chemotherapy. The lack of vasculature is a hallmark cause of hypoxia in solid tumours, yet many drugs depend on the cardiovascular system for their transport into the tumour. Different drugs have varying capacities to travel through cell layers, with larger drugs like doxorubicin, mitoxantrone, and paclitaxel showing particularly slow tissue penetration [260-263]. Many chemotherapies target rapidly dividing cells; hypoxia can limit cellular proliferation, or induce a quiescent state fueling chemoresistance [264-267]. Hypoxia creates broad changes in tumour dynamics that passively limit the efficacy of many therapies.
Hypoxia induces gene expression changes that actively cause adaptation to better allow cancer cells to resist the effects of chemotherapy [268, 269]. ABC transporters are a class of at least 48 highly conserved transmembrane proteins that protect cells from cytotoxic agents [268, 269]. In invasive breast cancer with lymph node metastasis, the expression of ABC protein P-glycoprotein (MDR1), the first multi-drug-resistance conferring protein discovered, is correlated with the presence of tissue hypoxia markers [270]. In three-dimensional culture, the upregulation of MDR1 is mediated by HIF1α. It was confirmed that this upregulation conferred drug resistance to the MCF7 cell line [271]. MDR1 is upregulated in breast CSCs from multiple cell lines in a HIF1α-dependent manner. Knockdown of HIF1α decreases MDR1 expression and dramatically reduces the resistance of these CSCs [272]. Hypoxia and HIF1α have been shown to regulate many pathways resulting in chemoresistance: AP1, MRP1, TMEM45A, PDGF, Bid, Bax, WNT, PI3K, NFkB, COX-2, c-Jun, Pim-1, and IAP-2 [273-283]. HIF1A, though important, mediates a subset of the effects of hypoxia. Some hypoxic responses are HIF1A independent. For example, transcription factor and signal transducer STAT3 increased chemoresistance in MDA-MB-231 by increasing expression of ABC transporters ABCC2 and ABCC6 independent of HIF1α. This effect was particularly notable in the stem cell fraction [284]. Modulation of protein synthesis in response to hypoxia-induced ER stress increased chemoresistance [285, 286]. Because hypoxia alters cancer cell phenotypes so broadly and through multiple interconnected pathways it has proven to be a tremendous obstacle to overcome.

1.3 NODAL

NODAL (Human: gene symbol: NODAL, NCBI gene ID: 4838; Mouse gene symbol: Nodal, NCBI gene ID: 18119) is a member of the TGF-β superfamily of proteins. The current full name of the protein is “nodal growth and differentiation factor.” This large family of structurally related proteins is characterized by proprotein secretion, activation by convertases mediated cleavage, a structural “cysteine knot” and a seventh cysteine residue that facilitates dimeric binding [287].

Nodal was discovered in the node of mouse embryos and has since been found to have numerous roles in development, embryonic stem cells, and carcinoma progression. Nodal
has three roles in the embryo: anterior-posterior axis formation, mesendoderm induction, and left-right asymmetry [288-293]. What is of particular interest is that Nodal is re-expressed in many cancers after being epigenetically silenced in most adult tissues. Two other important pieces of evidence inform our investigation of Nodal. First, Nodal maintains pluripotency in ESCs, suggesting that Nodal reemergence may be an important factor in breast cancer’s acquisition of phenotypic plasticity. Second, is that hypoxia stimulates Nodal expression, therein amalgamating Nodal into broader pluripotency and pro-survival signalling networks. For these reasons, we hypothesize a central role for Nodal in hypoxia-induced plasticity.

1.3.1 NODAL Signalling

Most of our understanding of vertebrate NODAL signalling comes from mouse developmental models. Once NODAL is secreted into the extracellular space, the 347 amino acid pro-protein is proteolytically cleaved into its mature 109 amino acid form by two subtilisin-like pro-protein convertases FURIN and PACE4 (Fig. 1.1) [294]. Following cleavage, the mature NODAL peptides homodimerize and signal by binding to activin-like kinase receptors type I (ALK4/7) and type II (ACTRIIB) [295-297]. Binding of Nodal to the type I receptor is facilitated by CRIPTO or CRYPTIC — two glycosylphosphatidylinositol (GPI)-linked epidermal growth factor-cysteine-rich Cripto-1/FLR1/cryptic (EGF-CFC) family proteins [297-299]. CRIPTO or CRYPTIC perform the secondary function of recruiting the type II receptors forming the active receptor complex. Though co-receptor expression is often necessary for NODAL signalling, some evidence suggests that CRIPTO-independent signalling is possible [297]. Stable receptor formation initiates signal transduction through the phosphorylation of receptor-regulated SMAD proteins (R-SMAD), SMAD2 and SMAD3 [291, 297, 300]. Phosphorylated R-SMADs bind to SMAD4 and translocate to the nucleus, however because of their relatively poor affinity for DNA they need to form complexes with transcription factors like FOXH1’, MIXER’, and p53 to regulate Nodal downstream genes like Goosecoid (Gsc)’, and Lefty1 [301-304]. Among the multiple processes regulated by NODAL invertebrates, the genes most consistently upregulated in all processes by NODAL are Nodal, Lefty, and Pitx2 [304, 305]. NODAL’s expression is governed by
Figure 1.1: The NODAL Signalling Pathway.

Pro-NODAL is cleaved by convertases into mature NODAL which then forms a homodimer that is stabilized by disulfide bond formation between the cysteines of the monomer’s growth factor cystine knots. CRIPTO facilitates the binding of the NODAL dimer to ALK4/7 and recruits ACTRIIB to form the active signalling complex. SMAD2/3 are phosphorylated, bind to SMAD4, and translocate to the nucleus to interact with transcription factors to regulate gene expression.
SMAD2/FOXH1 binding an enhancer in Nodal’s first intron creating a positive feedback loop [306]. In normal systems, a runaway effect is prevented by NODAL also upregulating its antagonist LEFTY and thereby creating a classic feedback loop [304]. LEFTYs are monomeric divergent members of the TGFβ superfamily that cause inhibition of NODAL signalling by binding NODAL or CRIPTO/CRYPTIC to prevent receptor complex formation [304, 307, 308]. The full extent of the relationship between NODAL and its antagonists is still actively being researched. NODAL regulation happens spatiotemporally. LEFTYs have a greater diffusion range than Nodal [304, 309]. This allows NODAL to exert stronger signalling close to its source, but as the distance from the source increases the relative strength of Lefty grows, and the inhibitory signal supersedes that of NODAL [172, 310, 311]. Different concentrations and durations of Nodal signalling will cause the expression of different sets of genes [312].

1.3.2 NODAL in Cancer

The discovery of NODAL in cancer resulted from an experiment that exploited the embryonic nature of the protein. Aggressive melanoma cells were injected into zebrafish embryos. These cells, but not poorly aggressive melanoma cells, induce ectopic axis formation phenocopying the effects of NODAL in developmental experiments [313, 314]. Using LEFTY, receptor inhibition, and NODAL knockdown strategies, NODAL was demonstrated to regulate tumour growth, clonogenicity, and cell fate [315]. Building on this original discovery, NODAL expression has been described in many cancers including breast, choriocarcinoma, endometrial cancer, glioma, glioblastoma, hepatocellular carcinoma, ovarian, pancreatic and prostate cancer [135, 316-331].

In breast cancer, the data establishes NODAL’s role in regulating invasion and migration in vitro, using transwell assays and 3D culture [317]. These data support the timing of NODAL expression in patients, which appears coincidently with invasive disease. NODAL is first detected by IHC in DCIS, an early stage non-invasive neoplasia, and increases dramatically during the progression to invasive DCIS [151]. These pro-tumourigenic phenotypes were regulated by ERK signalling and an increase of EMT [319]. Vascularization, as regulated by PDGF and VEGF, is also induced by NODAL expression. This result experimentally suggests a mechanism to explain the correlation
between NODAL expression and CD31 vascular staining [318]. Two separate groups report NODAL’s ability to upregulate proliferation and suppress apoptosis [317, 320]. The two groups provided accompanying analyses that came to complementary conclusions. Quail et al. demonstrated that NODAL is required for secondary site outgrowth, allowing micrometastases to form into macrometastases [317]. Strizzi et al. analyzed NODAL expression from 431 breast cancer patients and found that NODAL correlated with malignancy, dedifferentiation by Nottingham Grade, tumour stage and lymph node stage — these correlations were independent of intrinsic subtype [320]. Similar conclusions were reached by others for breast cancer, as well as eight other carcinomas, in a meta-analysis of 11 studies representing over 1000 patients. The clinical correlations found in these data were between Nodal and clinical grade (III+IV versus I+II), tumour size, and differentiation degree [332].

Work in other types of cancer may provide valuable insights that can further our understanding of NODAL’s role in tumourigenesis. Work in melanoma and breast cancer shows a distinct absence of endogenous NODAL inhibitor LEFTY expression in cell lines [151]. Reintroducing hESC derived Lefty represses NODAL. Introducing melanoma cells to hECS-conditioned matrix caused a re-differentiation event that resulted in the melanoma cells regaining MELAN-A expression [150, 151]. These were the first data to provide evidence for a NODAL-based re-differentiation therapeutic strategy. One set of data in prostate cancer suggests that Nodal may regulate phenotypes in a cell line-specific manner [330]. Though no mechanisms explaining the differences were presented, this does suggest that we should expect a heterogeneous response from cell lines in other cancers and potentially in patients should a targeted therapy be developed [330]. NODAL, being an embryonic protein, may directly regulate plasticity. This hypothesis is supported by data in pancreatic cancer in which NODAL regulates sphere formation, concomitant with an increase in a stem cell gene signature. NODAL expression also increases invasion and chemoresistance in pancreatic cancer, traits which are commonly observed in CSCs [328]. A study focusing on the interaction between adult liver stem cells and hepatoma cells suggests that in some tissues multipotent stem cells may act to suppress tumour formation through the expression of NODAL inhibitors. It may be worth exploring this concept in other tissues like the mammary gland and cycling
endometrium that exhibit appropriately controlled NODAL expression [333, 334]. In choriocarcinomas and trophoblast cells, NODAL signal transduction is mediated in part by β-arrestins and Ral GTPases [322]. Insights into regulatory pathways have the potential to help find targetable points in signalling cascades or to aid in the discovery of novel responsive genes.

1.3.3 Nodal in Stem Cells and Mouse

Patterning of the mouse embryo by Nodal is a meticulously regulated process that requires the establishment of signalling gradients resulting from the spatial expression of Nodal and its antagonists. NODAL’s first developmental function is in blastocyst development where it promotes the growth of the structure and blocks differentiation [290, 335]. Expression of NODAL in the epiblast signals to the extra-embryonic ectoderm (ExE) and the extra-embryonic (visceral) endoderm (VE) [336]. Nodal from the epiblast regulates gene expression and patterning in the ExE and induces NODAL expression in the VE [337]. NODAL signalling from the VE induces the expression of endogenous NODAL inhibitors, LEFTY1 and Cerberus (CER1), contributing to the specification of distal visceral endoderm (DVE) [289]. Secretion of the NODAL antagonists in the DVE is part of the signalling required for delineating the proximal-distal axis. Repression of NODAL in the epiblast results in the specification of the DVE to the anterior visceral endoderm (AVE) which is the first step in defining the anterior-posterior axis [338, 339]. Through coordinated cell movement of VE and epiblast cells, the AVE moves anteriorly, restricting NODAL expression to the epiblast’s posterior side [338-340]. Proximal posterior epiblast cells undergo EMT and begin to form mesoderm [289, 293, 337]. Another set of cells migrates toward the anterior of the epiblast. These cells have higher NODAL expression and form the primitive node, which in turn maintains the continued growth of the primitive streak [341, 342]. The cells from the ‘anterior streak’ specify into definitive endoderm [341]. Upon the completion of gastrulation NODAL expression is restricted to the node — the structure in which it was discovered and for which it was named. A combination of flow created by active cilia and left side expression of NODAL inhibitors creates another gradient that establishes the basis for the left-right asymmetry seen in organ development [302, 343].
Human embryonic stem cells are the best available model to study the dynamics of NODAL signalling in a non-pathological human system. Consistent with the expression of NODAL in the inner cell mass of the developing blastocyst, the cells from which stem cells are derived, NODAL maintains pluripotency in hESCs and blocks differentiation toward neuroectoderm lineages [344-347]. Neural commitment requires the loss of Nodal expression and the subsequent repression of OCT4 and upregulation of SOX1 [347]. To maintain pluripotency NODAL induces SMAD2/3 signalling that sustains the expression of \( \text{NANOG} \) by binding its promoter [348]. SMAD2/3 are also capable of interacting with \( \text{NANOG} \) to recruit DPY30 to the promoters of other genes [349]. In this way NODAL maintains H3K4me3 activating marks on genes required for pluripotency like \( \text{NANOG}, \text{OCT4}, \text{LEFTY1}, \) and \( \text{NODAL} \). NODAL also sustains these marks as part of bivalent promoters found regulating genes expressed in the primitive streak, mesoderm, and endoderm [349]. These data support the idea that NODAL can both maintain pluripotency and direct differentiation at different stages of development depending on the cellular context and that these different roles may be regulated through the coordination of different epigenetic pathways [349].
1.4 Translation

Translation of mRNA to protein is the most energy consuming process in the cell [235, 350]. It is unsurprising that such an intensive process is exceedingly well controlled. Translation provides a secondary means of regulating gene expression independent from changes in transcript abundance. The role of translation in gene expression is evident in the low concordance between the proteome and steady-state mRNA levels [351]. In times of stress, like hypoxia, rapid and extensive changes resulting from translational adaptation outnumber those changes resultant from transcription alone [352]. This adaptation is critical in cancer and many other cellular processes [353-355].

1.4.1 Translation Initiation

Because translation is so resource-intensive, much of the regulation of the process occurs early at initiation. Translation initiation requires the coordination of nearly a dozen different eukaryotic initiation factors (eIFs) to assemble the ribosomal subunits, initiator Met-tRNA\textsubscript{i}Met and mRNA [356]. The steps required for translation are the formation of the ternary complex, and subsequent formation of the 43s preinitiation complex; recruitment of the 43s complex to the 5' end of the mRNA; scanning the 5'UTR for the start codon (AUG); and assembly of the complete 80s ribosome (Fig. 1.2) [357].

At the beginning of this process eIF2 is bound to GDP. In this state eIF2 is incapable of binding Met-tRNA\textsubscript{i}Met [358]. The guanine nucleotide exchange factor (GEF) eIF2B exchanges the GDP for GTP allowing recruitment of Met-tRNA\textsubscript{i}Met and formation of the ternary complex [359]. eIF3, eIF1 and eIF1A maintain the 40s ribosomal subunit in a free state by preventing it from associating with the 60s subunit [360]. The recruitment of the ternary complex to this 40s ribosomal complex completes the formation of the 43s preinitiation complex. eIF2, the central mediator of this process, has three essential subunits: α, β and γ. We only have a partial understanding of the roles of each subunit. The α subunit contains a phosphorylation site that regulates the interaction between eIF2 and eIF2B preventing guanine exchange [361, 362]. This domain is essential to stress-induced translation regulation. The β subunit’s functions include regulating eIF2B and eIF5 binding as well as promoting the eIF2γ GTPase activity [363, 364]. Lastly, the γ
Figure 1.2: Translation Initiation

Translation begins with eIF4E bound to other eIFs recognizing the 5’m7GpppX cap. Next, the 43s preinitiation complex is recruited to the 5’ end of the mRNA by the cap binding complex. After recruitment to the cap scanning of the 5’UTR commences until the Met-tRNA reaches the start codon (AUG). Upon recognition of the start codon the eIF2α GTP is hydrolyzed and the 60s ribosome is bound to complete the translation competent 80s ribosome. The ternary complex is then recycled by eIF2B which exchanges the eIF2α bound GDP with GTP, followed by the subsequent recruitment of Met-tRNA (ternary complex). The ternary complex then binds the 40s ribosomal subunit to form the 43s preinitiation complex.
subunit forms the structural and enzymatic core of the complex, binding both GTP and Met-tRNA$_{\text{Met}}$ while also being responsible for hydrolyzing GTP [363, 365].

Concurrently, another set of eIFs prepare the mRNA for scanning. Activation of mRNA is accomplished by eIF4F, which comprises three eIFs: eIF4E, which binds the 5`m7GpppX cap, a structure found at the end of all transcribed eukaryotic mRNAs; eIF4A, an RNA helicase; and eIF4G, a scaffold protein that binds these proteins to other translation-regulating proteins while also increasing the helicase activity of eIF4A. The eIF4F complex unwinds the mRNA secondary structure preparing it for the 43s complex [366-369].

Scanning commences upon recruitment of the 43s complex to the mRNA cap [370]. eIF1, eIF1A, eIF4A, and eIF4G all contribute to the unwinding of the secondary structure required for scanning [369]. eIF1 reads the RNA sequence for AUG codons using context-dependent cues like the Kozak sequence or distance from the 5` end of the mRNA to determine the correct start site [371-373]. Once the correct start codon has been reached, eIF5 binds the β subunit of eIF2 triggering eIF2γ to hydrolyze GTP to GDP and thereby decreasing the complexes affinity for Met-tRNA$_{\text{Met}}$[363, 374]. Addition of the 60s ribosomal subunit and release of eIF1, eIF1A, eIF3 and residual eIF2–GDP is initiated by eIF5B. eIF2B replenishes the ternary complexes, and another series of eIFs separates the post-translation ribosomes into the free 40s and 60s pools [360, 373].

1.4.2 Regulating Translation at the Initiation Stage

Regulation of translation is a sophisticated process. Much like transcription, translation of each mRNA is regulated by unique sets of cis- and trans-acting elements. The sequence, length, and structure of the 5` UTR all effect translation [375-377]. Important sequence elements include uORF, 5` terminal oligopyrimidine tract (5`TOP), and GC rich (GGC)-repeat motifs [376, 378-380]. An important aspect of many of these features is that they are required for the differential regulation of translation under stress conditions as seen with length, uORFs, and 5`Top sequences [376, 377, 381, 382] These features likely
interact with different eIFs or with the same eIFs with different efficiencies in response to stimuli [234, 383-385].

Two themes permeate the role of translation in cancer. The first theme is that increases in the proteins required to initiate translation, eIFs, release important modulators of the cell cycle leading to uncontrolled growth [386-389]. The second theme is that in times of stress, cancer cells will limit translation to a subset of proteins that broadly promote survival, consequently leading to a more aggressive cancer.

Various changes in translation initiation have been demonstrated to have broad effects on pathogenicity. Early work showed that many individual eIFs are upregulated in cancer and that these changes correlate with cancer progression [390-393]. Regulation of translation is important for maintaining the balance between apoptotic and cell survival signals. Overexpression of eIFs is important in overcoming limitations on proliferation rate [394]. eIF4E increased chemoresistance and decreased apoptosis in fibroblasts with constitutively expressed c-myc, primarily through increasing the rate of cyclin D1 translation [389]. Li and colleagues showed that the reduction of eIF4E decreased cytochrome c release and endoplasmic reticulum-mediated apoptosis, implicating the increased translation of Bcl-XL as part of the mechanism [386]. Similar experiments utilizing antisense oligonucleotides to knockdown eIF4E in MDA-MB-231 cells decreased another Bcl protein, Bcl-2 [395]. The many roles of eIF4E has in the progression of cancer, the frequency of its upregulation, and its transforming potential in NIH 3T3 cells have identified it as a proto-oncogene [396, 397]. eIF4A1, a well-studied RNA helicase, has also been implicated in several cancers including breast, melanoma, hepatocellular carcinoma, and early stage non-small cell lung cancer [378, 390, 398, 399]. In breast cancer eIF4A1 expression remolds the translational landscape by upregulating other eIFs which ultimately changes gene expression patterns. With the increase in helicase activity, mRNA from oncogenic pathways with long, typically inefficient, 5’ UTRs are translated more efficiently. These mRNAs included TGF-β, Smad2, ARAF and the CDK1 cyclin activator CDC25B. Higher levels of eIF4A1 and lower levels of the eIF4A1 inhibitor PDCD4 are both predictive of poor clinical outcomes contributing to both our mechanistic and prognostic understanding of cancer.
Alterations in the ternary complex regulation show similar effects. eIF2α is upregulated in benign and malignant neoplasia, which increases cyclin D1, therein enhancing proliferation [400].

Maybe the most significant complicating factor that must be addressed with regard to translation and cancer is the role that repressing translation plays in cell survival. Microenvironmental factors are primarily responsible for decreasing translation and activating the translational stress response. Cells in internal regions of a tumour often lack the local vasculature necessary to deliver nutrients and oxygen. Such cells become stressed, triggering a decrease in global rates of translation to maintain energy homeostasis. One of the most potent mediators of this conservation is hypoxia [233, 401]. Two mechanisms are responsible for ensuring a global decrease in translation observed during stress. First, any number of stress responses converge on the eIF2α to cause its phosphorylation. In hypoxia this is often in response to endoplasmic reticulum stress, which causes eIF2α phosphorylation preventing the release of GDP; in this inactive state, ternary complex formation and recognition of the start site of translation is prevented. Second, multiple pathways also converge to inactivate mammalian target of rapamycin (mTOR) which leads to the dephosphorylation and activation of eIF4E binding protein (4E-BP), a translational repressor. 4E-BP binds to eIF4E preventing the formation of the cap-binding complex. Without eIF4E the translational complex cannot identify the 5′ cap [402]. Under stress, cap-dependent translation is suppressed. This process conserves valuable energy while inducing an adaptive stress response, such that transcripts encoding proteins required for survival can still recruit ribosomes by means of internal ribosome entry site (IRES) sequences in their 5′UTRs, alternate eIFs or changes in initiation dynamics cause by sequence motifs [402].

1.4.3 The Regulation of Translation in Cancer

One of the best characterized examples of stress regulating tumourigenicity is PERK–eIF2α–ATF4 signalling [403, 404]. This pathway is one of three mediators of the unfolded protein response, and PERK is one of the four kinases that phosphorylate eIF2α that compose the integrated stress response (ISR) [405-407]. Reduction of translation decreases reinitiation and pausing at the inhibitory ATF4 uORF thereby increasing
translational efficiency, which increases cellular ATF4 levels and causes the subsequent activation of its target genes [403, 408-411]. This pathway has been shown to be important in angiogenesis, migration, survival, EMT, and metastasis [403, 408-410]. Hypoxia-induced PERK-dependent translational regulation governs the gene expression of a number of important cancer phenotypes such as angiogenesis, cellular adhesion, and cell growth and signal transduction; how these changes affect cancer phenotypes is an open research question [282, 410, 412].

Translation inhibition by nutrient deprivation or chemical inhibition increases phenotypic plasticity attendant with chemoresistance [413]. It also appears that the PERK eIF2α pathway activates in response to treatment of some cancers which in turn increases therapy-resistant gene expression [414]. Furthermore, higher levels of PERK predicted poor response to radiotherapy meaning that both the activation and the increased expression of pathway components are radioprotective [414]. EMT and eIF2α phosphorylation have a reciprocally activating relationship that promotes plasticity, CSC formation and subsequent tumourigenesis [410, 412]. A notable insight in these papers is that activation of the PERK eIF2α pathway sensitizes cells to further UPR stress [410, 412]. These observations hold true in non-transformed mouse ESCs. Treatment with BMP4, leukemia inhibitory factor (LIF), and Salubrinal inactivate eIF2α resulting in the increased translation of uORF containing mRNA like Nanog, and c-Myc [413]. The phosphorylation of eIF2α regulates plasticity and quiescence in muscle stem cells [415].

mTOR is the master regulator of numerous processes, many of which have a component of their regulation resultant from translation, yet not all changes resulting from mTOR can be attributed to translation. Likely due to the far-reaching signalling effects of mTOR, its role in tumour progression, especially regarding plasticity, is unclear. These conflicting results are well summarized in a review by Xia and Xu [416]. Embryonic systems may provide a simpler system to study the ‘default role’ of mTOR-mediated translation restriction independent of confounding variables like dysregulation of related pathways, interfering mutations, and cancer or stage specific effects. Multiple experiments in mouse ESCs link lower endogenous mTOR activity or experimentally suppressed mTOR activity to the support and maintenance a pluripotent phenotype. One
means of ruling out non-translation mTOR effects is to focus on one of the downstream proteins that regulate translation, namely 4E-BP1. In mouse ESCs YY2 is responsible for maintaining pluripotency and for later directing lineage-specific differentiation much like Nodal. The balance of regulation needed for the maintenance of pluripotency results from a combination of splicing Polypyrimidine tract-binding protein 1 (PTBP1)- and 4E-BP1-mediated translational repression [417]. In these experiments the absence of translational repression decreases a comprehensive pluripotent gene signature. Similarly, these types of observations hold true in multipotent progenitor cells [418]. Though the evidence is scarce, mammary stem cells seem to also require decreased mTOR activity for maintenance [419]. These data clearly indicate that the mechanism exists for mTOR inhibition and the ensuing translational repression to regulate pluripotency through the selective translation of pluripotency factors. An important addition to these data come from iPSCs in which 4E-BP1 and low mTOR activity support the reacquisition of plasticity [420]. The subtlety of these systems is best represented by He et al. where mTOR inhibition improved reprogramming efficiency and the presence of Sox2 subsequently decreased mTOR expression. But, the improvement in reprogramming efficiency was lost if mTOR inhibition was prolonged [421].

Both EMT and plasticity are emerging as cancer phenotypes driven by translational reprogramming. Key mediators of these phenotypes have demonstrated enhanced expression that results from changes in the efficiency of their translation. This concept is consistent with the idea that stem cells and differentiation require specific translational programs [417, 422, 423]. Relationships first discovered in stem cells like that of Lin28 mediating the translation of OCT4 have been reproduced in cancer [423, 424]. The observation of this phenomenon in both systems increases both the reliability and our ability to interpret these data. The role of translation in decisions of cell fate are proving to be a deeply complex and highly regulated system of processes. Snail a key mediator of EMT is enhanced translationally by multiple RNA binding proteins. When the tumour suppressor SCRIBBLE is knockdown HuR accumulates in the cytoplasm where it binds to the AUrich elements found in the 5´UTR of SNAIL1 resulting in an increase in SNAIL1 translation. In another study translation of an EMT signature that includes SNAIL1, TWIST, and ZEB2 enhanced by the overexpression of YB1 an important translational
regulator that controls IRES mediated initiation frequently overexpressed during neoplastic progression [425, 426]. In pancreatic ductal adenocarcinoma ZEB1 translation is enhanced by the collagen microenvironment induced MNK activation resulting in eIF4E phosphorylation. In this model MNK inhibition prevent CSC formation along with an increase in E-cadherin and a decrease in Vimentin expression suggestive of broader reprogramming resulting from eIF4E phosphorylation [427]. ZEB1’s translation is also regulated by polyadenylation in response to genotoxic stress demonstrating the role translation plays in facilitating a plastic-EMT response to stress conditions [428].

Overexpression of eIFs suggest that the capacity for high levels of translation and proliferation promote tumourigenicity and that these changes can occur early in transformation [397]. The current literature also suggests that a decrease in translation contributes to tumourigenicity through the preservation of phenotypic plasticity. The duality of roles may represent heterogeneous subpopulations within the tumour analogous to tumours possessing both proliferative and non-proliferative cells, treatment-sensitive and treatment-resistant cells, and cells with lower or higher levels of tumour initiation potential. It is precisely this utilization of heterogeneous phenotypes that allows for the adaptation to microenvironmental factors and cellular niches, including stresses, that facilitate tumour cell survival while simultaneously sustaining high proliferation characteristic of the bulk of tumour cells.
Figure 1: Stress-Induced Translational Inhibition Increases Tumourigenic Phenotypes via Plasticity.

A model of how stresses, like hypoxia or chemotherapy, can affect the mTOR/4E-BP1 or the PERK/eIF2α pathway resulting in plasticity and tumourigenicity. The experimental methods of modeling these stresses include: mTOR inhibition (INK128), 4E-BP1 overexpression, ER stress (AZE—azetidine-2-carboxylic acid), blocking eIF2α, blocking mTOR, and blocking AMPK. By allowing ternary complex recycling in the presence of eIF2α phosphorylation, integrated stress response inhibitor (ISRIB) can prevent the eIF2α dependent adaptive response.
1.5 Thesis Rationale, Hypothesis, and Aims

The theoretical basis for this thesis derives from the lack of concordance between NODAL mRNA and protein expression in response to hypoxia, the known role of translational regulation as an adaptive response to hypoxic conditions, and the potential role that translation can play in the maintenance and acquisition of pluripotency [135]. Because translation alters global protein expression, it is likely that the phenomenon that we have observed in NODAL applies to other regulators of plasticity, as well as those proteins necessary for stress-specific responses. The concept that the activation of these pathways is not exclusive to hypoxia is supported by data that demonstrates that translation inhibition is part of cancer’s pro-survival response to chemotherapy [429, 430]. Therefore, I hypothesize that breast cancer adapts to stresses, including hypoxia and chemotherapy, through selective translation of mRNA to produce plastic and pro-survival phenotypes. Subsequently, the inhibition of said translational adaptation should result in the diminution of adaptive responses. To address these questions, my first aim will be to investigate how hypoxia alters plastic phenotypes and to establish post-transcriptional mechanisms that support these responses (Chapter 2). As there are two primary pathways, mTOR/4E-BP1 and PERK/eIF2α that regulate the adaptive translational response, I will explore each of these separately. My second aim is to characterize the role of mTOR and 4E-BP1 on translational stress response and how these promote plasticity (Chapter 3). The prospect that Nodal in consort with other pluripotency factors and plastic phenotypes like EMT is regulated at the level of translation may provide new insights into the acquisition and coordination of these features. Furthermore, I will test whether suppression of stress response pathways can reverse the adaptive acquisition of plasticity. The third aim sets out to determine if ISR-based cellular adaptation increases plasticity and how inhibition of this response can enhance susceptibility to chemotherapy and decrease plasticity (Chapter 4). Figure 1.3 is a diagrammatic overview of how these pathways will be experimentally tested. Through these experiments I seek to demonstrate that stress increases plasticity in vivo and that these effects promote tumourigenicity contributing to treatment failure. Through these findings, I intend to establish the role that restricting translation plays in mounting a
concerted stress response that includes the acquisition of phenotypic plasticity while demonstrating for the first time, that these responses may be broadly targetable within the framework of current first-line therapies.
References


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Chapter 2

2 The Characterization for Translation’s Role in the Acquisition of Plasticity

2.1 Introduction

For years, compelling evidence has amassed that plasticity and dedifferentiation drive cancer progression. This concept holds true across multiple tumour types, stages of the disease, and is supported by an ever-growing list of implicated pathways and mechanisms. These concepts have been extensively reviewed [431-433]. Significant advancements in our understanding of these processes have been achieved through the identification of many drivers of cancer plasticity like NODAL, OCT4, NANOG, and EMT promoting transcription factors [106, 117, 151]. Phenotypic plasticity seems to be intrinsically linked to tumourigenic traits like metastasis and chemoresistance [265]. The work of many laboratories supports the concept that the expression of stem cell genes drives phenotypes other than plasticity like radioresistance, chemoresistance, or metastasis, but very few studies have attempted to address the higher order mechanisms that coordinate such a network of responses [92, 434, 435]. There seem to be some innate features, like the quiescence of CSCs, which derive directly from the expression of stem cell proteins, which may account for some of the correlation between these [266]. I intend to demonstrate that hypoxic stress can induce extensive reprogramming of the translatome. These changes cause parallel activation of pathways that support plasticity and survival, like those induced by ATF4. When adaptations resulting from translational reprogramming are taken together with the known changes induced by hypoxia to aspects of cellular physiology like metabolism and energy conservation, it is evident that hypoxia can be a central driving force of tumourigenic progression and survival-promoting adaptations. Building on the work of Quail et al. who demonstrated that hypoxia increases NODAL in breast cancer without the expected upregulation of NODAL mRNA and the current evidence that NODAL causes a dedifferentiated plastic phenotype, I hypothesize that hypoxia will increase plasticity through broad changes in gene
expression, including the upregulation of NODAL, that will require specific mRNAs to escape from the global translational repression incited by hypoxia [135, 151].

Comprehensive transcriptional and translational changes in gene expression induced by hypoxia bolster CSCs and plasticity in breast cancer [236, 436, 437]. Determining translational efficiency through methods such as polysome profiling, can the lack of concordance between translation and steady-state mRNA levels, having been found to more reliably correlate with protein abundance than many other quantification methods [351, 438-440]. More importantly though, changes in translational efficiency may be a targetable shared mechanism for survival, chemoresistance and plasticity. Translation may then become a rational explanation for the concurrent phenotypes of chemoresistance and plasticity so often observed in cancer stem cells.

Most of the known mechanisms for changes in translation derive from the sequence, length, and structure of 5′UTRs [441]. These include putative internal ribosome entry sites (IRESs), 5′TOP sequences, or uORFs [380, 442, 443]. In hypoxia hundreds of responsive genes change their transcriptional start sites, thus altering the composition of their UTRs. This process was demonstrated to be highly cell type dependent, though there were higher levels of similarity among more closely related cell types [441]. Examination of the CAGE TSS HMM Encode/Riken data set for potential TSSs shows putative alternative TSSs for many pluripotency factors and EMT markers. It is conceivable that these could be used to facilitate selective translation of key mRNAs in response to hypoxia.

2.2 Results

2.2.1 Hypoxia Regulates Plasticity in Breast Cancer

CSCs are a significant impediment to effective clinical treatments. These cells are plastic, treatment resistant, and are highly malignant [36, 80]. It is unsurprising than that their presence has prognostic value predicting poorer clinical outcomes [36, 80]. Because of the rarity of these cells and the variable expression of stem cell surface markers, a
Figure 2.1: Hypoxia Increases Sphere Formation in Breast Cancer Cell Lines.

(a) T47D cells grown at 20% (normoxia), 0.5% (hypoxia) oxygen, or hypoxia in the presence of ALK5 inhibitor SB431542 for 24h before being plated from a single cell suspension into non-adherent 96 well plates. Each well received, on average, ten cells per well. Cells were given three weeks at 20% oxygen to form spheres. Cells showed an increase in tumoursphere formation (p<0.005, n=3). Bars represent the mean tumoursphere count ± SD. The letters indicate a significant difference, unpaired t-test. (b) Phase contrast images of spheres derived from cells pre-exposed to 20% O$_2$ or 0.5% O$_2$ (500µm scale bar). (c) SUM149 cells grown at 20% (normoxia), 0.5% (hypoxia) oxygen, or hypoxia in the presence of ALK5 inhibitor SB431542 for 24h before being plated from a single cell suspension into non-adherent 96 well plates. Ten cells per well were plated. Spheres were given three weeks at 20% oxygen to grow. SUM149 cells increase tumoursphere formation (p<0.01, n=3). Bars represent the mean tumoursphere count ± SD. Different letters indicate a significant differences via, ANOVA p<0.05. (d) Phase contrast images of spheres derived from cells pre-exposed to 20% O$_2$, 0.5% O$_2$, or 0.5% O$_2$+SB (500µm scale bar).
consistent clinically relevant biomarker across all stages of breast cancer has yet to be identified. To overcome these limitations, we employ sphere forming assays as a functional measure of CSCs. This assay has also been tested for its clinical value [444-446]. The rate of sphere formation correlates with progression to metastasis and has been validated for use within clinical and pre-clinical research, which has led to their inclusion in trials for mTOR inhibitors like INK128 (ClinicalTrials.gov Identifier: NCT0213318) [447-449]. The formation of spheres requires multiple cellular phenotypes such as anoikis inhibition, and metabolic alterations, hypoxic adaptations, ROS resistance. These phenotypes are have been linked to plasticity and CSCs, however, it is difficult to determine if these changes are intrinsic aspects of cancer stem cells are coincidently expressed phenotypes [436, 450-452].

To model whether microenvironmental stresses increase the abundance of CSCs, we measured sphere formation in response to hypoxia in the poorly metastatic breast cancer cell lines T47D (Fig. 2 a, b) and SUM149 (Fig. 2 c, d). After cells were grown for 24h in either normoxia (20% O₂) or hypoxia (0.5% O₂), single cells were plated at 10 cells per well for the T47D line and 10 cells per well for the SUM149 line into 96-well non-adherent plates and left to grow for 3 weeks. In both cell lines a 24h hypoxia treatment significantly increased sphere formation. In T47D cells the sphere formation rate increased 2.2-fold and in the SUM149 cell line sphere formation increased 2-fold. Administration of the ALK5 inhibitor SB431542 (Hypoxia+SB) prevents the induction spheres by hypoxia, likely by blocking the NODAL signalling which increases in response to hypoxia [135]. Previously, it was shown that NODAL regulates many pro-tumourigenic phenotypes through EMT [319]. We hypothesized that these pathways would be upregulated by hypoxia in breast cancer cells. To test this T47D cells treated for 24h at 20% O₂ and 0.5% O₂ were compared using RNA-seq. The results of the gene set enrichment analysis (GSEA) are reported in Table 2.1. In the GSEA hypoxia was the most activated pathway. Though this was expected it serves as a good indication that T47Ds are a good model for these experiments as their response to hypoxia is robust. The third most upregulated pathway was EMT. The EMT gene signature was upregulated with a degree of certainty of q.val = 4.11x10⁻⁷ as determined by the Benjamini-Hochberg method and adjusted for false discovery rate.
Table 2.1: Top 5 Upregulated Gene Set from KEGG Gene Set Enrichment Analysis (GESA) of T47D Cells Exposed to Hypoxia.

The five most perturbed pathways in T47D cells treated with 0.5% O2. GAGE mean t-statistic, p-value and gene set size are reported as determined by GSEA. Adjusted p-value (FDR) of 0.05 was used as a cut-off (p-value adjusted for multiple tests using the Benjamini-Hochberg method). Subsequent gene set analysis used the GAGE package for R. All genes and their expression values relative to normoxia, regardless p-value, were used as input and hallmark gene sets from Molecular Signatures Database were used (n=3).

<table>
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<th>p.val</th>
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<td>113</td>
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Figure 2.2: Hypoxia Activates a Pluripotent Transcriptional Signature in a Cell Line Specific Manner.

(a) Real time RT-PCR analysis of MDA-MB-231 cells for *ATF4*, *OCT4*, *SLUG* (*SNAI2*), *TWIST1*, and *VEGF-A*. MDA-MB-231 cells were cultured in 20% or 0.5% oxygen for 24h. Hypoxia samples were normalized to paired normoxia samples and represented as log2 fold change (n=3). *OCT4* (p=0.05), *SNAI2* (0.028) and *VEGF-A* (p=0.0237) are all increased in MDA-MB-231 cells in response to hypoxia as indicated by the asterisks (*, p<0.05). (b) Real time RT-PCR analysis of MCF7 cells for *ATF4*, *OCT4*, *SOX2*, *TWIST1*, and *VEGF-A*. MCF7 cells were cultured in 20% or 0.5% oxygen for 24h. Hypoxia samples were normalized to paired normoxia samples and represented as log2 fold change (n=3). *ATF4* (p= 0.0007) and *VEGF-A* (p=0.0274) are altered in MCF7 cells in response to hypoxia as indicated by asterisks (*, p<0.05). All bars represent ± SD relative to the 20% O2 control. Significance was determined using paired Student’s t-tests.
EMT is a contributing factor to nearly every step in the metastatic cascade [111-116]. EMT also represents one method of acquiring phenotypic plasticity. The re-expression of NODAL and other stem cell factors represents a second mechanism for the reemergence of plasticity. Using real time RT-PCR, we determine whether 24h of hypoxia elicits a gene expression response that includes both EMT and pluripotency factors. Included among these are *ATF4*, a gene whose expression is controlled at the level of translation, and *VEGF-A*, a gene that is upregulated both transcriptionally and translationally in response to hypoxia. MDA-MB-231 cells, which are highly aggressive, invasive and dedifferentiated, show a transcriptional increase in known plasticity genes in response to 24h of hypoxia resulting in the increased expression of *OCT4*, *SNAI2*, and *VEGF-A* (p<0.05, n=3) (Fig. 2.2 a). In the less aggressive MCF7 cells there is no transcriptional response in the plasticity genes *OCT4*, *SOX2* and *TWIST1*. There is a small but significant transcriptional down-regulation of *ATF4*. Similar to the MDA-MB-231 cells, MCF7 cells upregulate *VEGF-A* (p<0.05, n=3) (Fig. 2.2 b). We investigated two other breast cancer cell lines T47D and SUM149. To determine if there were significant differences between short and longer-term responses to hypoxia T47D cells (Fig. 2.3 a) and SUM149 cells (Fig. 2.3 b) were left untreated (0h) or treated for 6h or 24h in 0.5% O2. The mRNA expression was analyzed by real time RT-PCR for three stem cell genes *NANOG*, *OCT4*, *SOX2* and an EMT transcription factor *SNAIL* (*SNAI1*). In the T47D cells *NANOG* and *OCT4* show transcriptional upregulation at 6h but only the *OCT4* expression is maintained at 24h (p<0.05, n=3). The SUM149 cells increased the expression of *NANOG* and *OCT4* at 6h and 24h, while *SNAIL* mRNA abundance is decreased at 6h and 24h (p<0.05, n=3). Though still below control levels *SNAIL* mRNA begins to recover after 24h of hypoxia treatment (p<0.05, n=3).

### 2.2.2 NODAL Drives the Acquisition of CSC Phenotype

Having observed cell line dependent differences in hypoxia induced expression of genes that drive plasticity, we sought to determine if NODAL was capable of promoting CSC formation, NODAL being a gene previously determined to be regulated by hypoxia [135]. To do this, SUM149 cells were transfected with an empty vector, a GFP containing vector, or a vector containing NODAL, via lentiviral transduction (Fig. 2.4 a).
Figure 2.3: Hypoxia Activates a Plasticity Supporting Gene Transcriptional Signature in Some Cell Lines.

(a) Real time RT-PCR analysis of T47D cells for NANOG, OCT4, SNAIL (SNAI1), and SOX2. T47D cells were cultured in 20% or 0.5% oxygen for 6h and 24h. Hypoxia samples were normalized to paired normoxia samples and represented as log2 fold change (n=3). NANOG and OCT4 are increased in T47D cells in response to 6h in hypoxia and OCT4 remained upregulated at 24h as indicated by the asterisks (*, p<0.05). (b) Real time RT-PCR analysis of SUM149 cells for NANOG, OCT4, SNAIL (SNAI1), and SOX2. SUM149 cells were cultured in 20% or 0.5% oxygen for 24h. Hypoxia samples were normalized to paired normoxia samples and represented as log2 fold change (n=3). NANOG and OCT4 are increased in SUM149 cells at both hypoxia time points and Snail is decreased as indicated by asterisks (*, p<0.05). All bars represent mean ± SD relative to the 20% O2 control. Significance was determined using one-way ANOVA.
Figure 2.4: Hypoxia Increase CD44\textsuperscript{high}/CD24\textsuperscript{low} Stem-like Breast Cancer Cells in the SUM149 cell line.

(a) SUM149 cells were stably transfected with an expression vector containing Nodal, GFP or an Empty vector and were assessed for the number of CD44\textsuperscript{high}/CD24\textsuperscript{low} cells. Conjugated CD44 (PE) and CD24 (FITC) antibodies were used to stain the cells before being counted by flow cytometry. The cells displayed an increase in this subpopulation in response in Nodal expressing cells (mean fold change ± SD, p<0.01, n=3). Significance was tested using one-way ANOVA comparing all groups to Nodal with Bonferroni and Holm post-hoc test. Representative scatter plots discriminating subpopulations as defined by cell surface markers CD44-PE and CD24-FITC. (b) SUM149 cells were exposed to hypoxia (0.5% O2) for 24h and the abundance of CD44\textsuperscript{high}/CD24\textsuperscript{low} cells was measured by flow cytometry. The cells displayed an increase in this subpopulation in response to rhNodal (mean fold change ± SD, p<0.001, n=3). Representative scatter plots discriminating subpopulations as defined by cell surface markers CD44-PE and CD24-FITC. Isotype and fluorescence minus one controls were performed (not shown). Significance was determined using paired Student’s t-tests.
Figure 2.5: rhNodal Increase CD44\textsuperscript{high}/CD24\textsuperscript{low} Stem-like Breast Cancer Cells.

(a) T47D cells were treated with 100 ng/mL of rhNodal for 24h to assess changes in the number of CD44\textsuperscript{high}/CD24\textsuperscript{low} cells. Conjugated CD44 (PE) and CD24(FITC) antibodies were used to stain the cells before being counted by flow cytometry. The cells displayed an increase in this subpopulation in response to rhNodal (mean fold change $\pm$ SD, $p=0.0065$, $n=6$). (b) MCF7 cells received 100 ng/mL of rhNodal for 24h to assess their changes in the number of CD44\textsuperscript{high}/CD24\textsuperscript{low} cells. The cells displayed an increase in this subpopulation in response to rhNodal (mean fold change $\pm$ SD, $p=0.008$, $n=6$). (c) SUM149 cells underwent the same treatment as (a) and (b) and the abundance of CD44\textsuperscript{high}/CD24\textsuperscript{low} cells was measured by flow cytometry. The cells displayed an increase in this subpopulation in response to rhNodal (mean fold change $\pm$ SD, $p=0.0014$, $n=6$). Representative scatter plots discriminating subpopulations as defined by cell surface markers CD44-PE and CD24-FITC. Isotype and fluorescence minus one controls were performed (not shown). Significance was determined using paired Student’s t-tests.
CSCs were measured by flow cytometry to enumerate the cells expressing CD44 that also had low expression of CD24 (CD44^{high}/CD24^{low}). Overexpression of NODAL doubled the number of CD44^{high}/CD24^{low} cells, though this difference failed to reach a level of significance greater than 0.05 (p=0.087, n=3). The effects of NODAL are much more constrained than those of hypoxia. Twenty-four hours of exposure to hypoxia (0.5% O_2) increased the stem cells from an average of 1.5% of the total to 33.6% (p<0.05, n=3) (Fig. 2.4 b). These data suggest that though NODAL may contribute to the expansion of CSCs by hypoxia the broad changes brought about by this stress are far more potent inducers of CSC formation. Three breast cancer cell lines — T47D, MCF7 and SUM149 (Fig. 2.5 a, b, c) were tested for the ability of 100ng/mL of recombinant human NODAL (rhNODAL) to induce CSC formation measured as the percent of the total population expressing the CD44^{high}/CD24^{low} phenotype. In all cases NODAL induces a modest increase in CSC formation (p<0.01, n=6). Figure 2.5 d shows the quadrant gating set according to fluorescence minus one (FMO) controls as well as a representative set of plots for SUM149 cells. Exogenous and endogenous NODAL are capable of increasing CSC formation. These data are in concordance with a model of NODAL that could signal in both an autocrine and paracrine manner [330, 453].

Tumours from three different models were used to determine if NODAL expression is localized to hypoxic cells. SUM149, patient-derived-xerograph (PDX) 401, and PDX574 tumours were implanted into the mammary fat pad of non-obese diabetic scid gamma (NSG) mice and allowed to grow to 10mm. Single cells were isolated from these tumours using the MACS Tumour Dissociation Kit. Once cleared of cell debris the cells were fixed and permeabilized, and then stained for CA9 and intracellular NODAL (Fig. 2.6). CA9 is a well characterized endogenous marker for tumour hypoxia which we can use to identify prospective hypoxic cells [454, 455]. Using CA9- and NODAL-conjugated antibodies we gated on the top 50% of CA9 expressing cells. A ‘not gate’ was used to capture all cells not contained within the CA9^{high} gate. These are the CA9^{low} cells as seen in the second histogram of Figure 2.6 d. The NODAL expression of the CA9^{high} (light blue) and CA9^{low} (yellow) cells are shown in the third histogram of Figure 2.6 d. The median NODAL florescence of the two CA9 expression groups for SUM149, PDX401,
Figure 2.6: NODAL High Cells from Tumours Have Higher CA9 Expression.

Single cells were extracted from (a) SUM149, (b) PDX401 and (c) PDX574 tumours grown in NSG mice. Cells were fixed and permeabilized to allow for staining using FITC conjugated CA9 antibody, and APC conjugated NODAL antibody. (a,b,c) The differences in the median NODAL fluorescence in each tumour between cells expressing relatively high or relatively low levels of CA9. (d) Histograms of CA9 FITC expression and NODAL APC expression of cells from PDX401 tumours. Differential expression of NODAL in CA9\textsuperscript{high} and CA9\textsuperscript{low} populations. The CA9\textsuperscript{high} gate contains cells represented in blue on the NODAL histogram and the remaining cells to the left of the gate are represented in yellow (CA9\textsuperscript{low}). All bars represent average ± SD relative to the 20% O2 control. Significance was determined using Student’s t-test. Asterisks (*) indicate a significant difference in nodal expression between CA9\textsuperscript{low} and CA9\textsuperscript{high} groups (p<0.05, SUM149 n=6, PDX401 n=6, and PDX574 n=4).
Figure 2.7: Nodal’s Stability is Increased in Hypoxia as Quantified in Cyclohexamide-treated T47D Cells.

(a) T47D breast cancer cells, transfected with a Myc-DDK-tagged Nodal expression, were cultured for 24h in 20% O₂ (normoxic) or 0.5% O₂ (hypoxic) conditions before being treated with cycloheximide to arrest translation for 30min to 24h. Nodal protein abundance was assayed by immunoblotting using an anti-FLAG antibody followed by quantification using densitometry and normalization to 0h (n=3). Protein stability was tested using a 24h cycloheximide treatment of 10µg/mL. (b) Representative blots show an increase in stability of Nodal in hypoxia. Bars denote the mean fold change ± SD. Asterisks (*) indicate a significant difference (p<0.05).
PDX574 tumours are shown in Figure 2.6 a, b, and c, respectively. In all three tumour types there is a clear relationship between the CA9 and NODAL expression (Fig. 2.6 a (n=6), b (n=6), c (n=4)). The breast cancer cell line SUM149 showed the greatest increase in NODAL expression between CA9\textsuperscript{high} and CA9\textsuperscript{low} groups. NODAL is most highly expressed in cells that are also expressing high levels of the hypoxia marker CA9 providing further evidence that NODAL is upregulated by hypoxia.

### 2.2.3 The Post-Transcriptional Regulation of Plasticity

Though it is becoming clear that NODAL and hypoxia can promote CSC formation it is still unclear how NODAL is being upregulated by hypoxia. It has been shown in other systems that NODAL signaling is regulated by protein stability [453]. To examine the effects of hypoxia on protein stability, T47D cells were transfected with a MYC-DKK-tagged NODAL pCMV6 vector and selected for stable expresses with G418 at 500µg/mL. Cells were grown for 24h in normoxic or hypoxic conditions, as previously described, at which point they were treated with cycloheximide to arrest de novo protein synthesis. The cells were lysed, and protein was extracted at 0h, 30min, 1h, 2h, 3h, 6h, 12h, and 24h. NODAL abundance was quantified by western blot analysis using an anti-FLAG antibody (Fig. 2.7 b) followed by quantification using densitometry and normalization to 0h (Fig. 2.7 a) (n=3). Under these conditions NODAL is more stable in hypoxia. Stability may help to explain NODAL’s expression in hypoxia, but without a source of new protein it seems insufficient to fully reconcile the upregulation of NODAL.

Using hypoxia treated T47D cells we confirmed that NODAL is not transcriptionally upregulated by hypoxia. To do so we quantified NODAL using real time RT-PCR, then calculated the abundance of NODAL transcripts in 2µg of RNA using a standard curve derived from a dilution series of a linearized NODAL vector. This process allowed us to accurately calculate the NODAL copy number in a sample (Fig. 2.8 a). NODAL’s transcription decreases in hypoxia between 1h and 24h, with the greatest suppression happening at 3h and expression beginning to recover at 24h (p<0.05, n=3). NODAL protein abundance increases during the same time course as measured by western blot.
Figure 2.8: Nodal Protein Increase in Hypoxia as the Absolute Copy Number of Nodal Transcript Decreases.

(a) Total copies of Nodal mRNA are calculated using a dilution series of a linearized Nodal vector containing known copy numbers ranging from 1 to 1x10⁹ and plotting the quantities against CT values to create a standard curve. Error bars represent the mean ± SD. Different letters indicate a significant difference (p<0.05, n=3). The least squares line of best fit (R²=99.77) was used to calculate the total copy number of Nodal in 2µg of RNA. (b) Representative western blot of total cell extract probed with anti-Nodal antibody for a hypoxia time course (0h, 1h, 3h, 6h, 12h, 24h). β-Actin is used as a loading control.
Figure 2.9: Translation is Globally Down-Regulated in Response to Hypoxia.

(a) MCF7 and (b) T47D cells cultured at 20% (Normoxia) or 0.5% O2 (Hypoxia) for 24h were then treated with cycloheximide (0.1 mg/mL) to arrest translation. Polysomes were fractionated by ultracentrifugation. Absorbance at 256nm was continuously monitored during the fractionation process. The polysome curves represent the total amount of ribosomes and polysomes present in each sample. The total volume of actively translating ribosomes is depressed in hypoxia in both T47Ds and MCF7s as shown by the fold change in 80s ration between normoxia and hypoxia.
Figure 2.10: Quality Control of Polysome Profiles.

A sucrose gradient polysome profile of T47D cells, from which four samples were extracted to measure for the enrichment of ribosomes. The fraction positive for ribosome enrichment is marked with an asterisk (*) and those lacking enrichment are indicated by (¤). Fractions show discernable characteristics of a polysome profile including 40s ribosome peak, 60s ribosome peak and regions of differential polysome binding. Each fraction was analysed by testing 4 technical replicates by LCMS/MS. Tryptic peptides were identified using UniProt FASTA database. Some ribosomal proteins and many translation initiation complex proteins are present in the fraction associated with the 60s ribosome peak.
Table 2.2: LCMS/MS Six Most Abundant Proteins Based in Polysome Fractions in Hypoxia and Normoxia.

Ultracentrifugation of cycloheximide treated cells is sufficient to enrich ribosomal proteins as detected by LCMS/MS. Common contaminants like Keratin and Trypsin were removed from the list.

<table>
<thead>
<tr>
<th>Normoxia</th>
<th>Hypoxia</th>
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<tr>
<td>60S RIBOSOMAL PROTEIN L7.</td>
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<td>60S RIBOSOMAL PROTEIN L6.</td>
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</tr>
<tr>
<td>40S RIBOSOMAL PROTEIN S3.</td>
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</table>


These data together demonstrate that transcription is an insufficient explanation for the upregulation of NODAL in hypoxia.

Given that hypoxia introduces significant restrictions on energy consumption and metabolism we tested to see if breast cancer cells adapt to hypoxia by restricting translation. For this MCF7 (Fig. 2.9 a) and T47D (Fig. 2.9 b) cells were tested. Both cell lines were treated for 24h at 0.5% O₂ and compared to untreated controls. For the MCF7 cells (Fig. 2.9 a) there is a clear decrease in the amount of RNA found in the polysome portion of the profile. This is summarized in the global translation bar graph as the fold change in mean absorbance of 80s ribosomes in the polysome portion of the absorbance curve. These same observations hold true in the second breast cancer cell line T47Ds. Poorly metastatic breast cancer lines adapt to hypoxic stress in part by reducing protein synthesis. To ensure the purity of the polysomes, portions of the T47D polysome profile were analyzed by liquid chromatography tandem mass spectrometry of the four samples tested the sample taken from the polysome portion of the profile (Fig. 2.10 (*)) was the most enriched for ribosomal proteins when compared to other fraction or whole cell extracts. This was true in normoxia and hypoxia (Table 2.2).

There are two pathways that have been identified as central regulators of translation in response to a broad range of stresses, both of which are essential to the downregulation of translation required for adaptation to hypoxia. These are the mTOR pathway and PERK/eIF2α pathway. To see if these pathways were plausible mechanisms regulating hypoxia-induced plasticity, we first established that these pathways were active in response to hypoxia in our breast cancer cell lines — T47D (Fig. 2.11 a) and MCF7 (Fig. 2.11 b). In parallel we tested the hECS line H9 (Fig. 2.11 c). Comparing the cancer lines to a non-transformed cell line helps to establish the dynamics of a normal hypoxic response. Two mTOR phosphorylation sites, Ser 2481 and 2448 were tested using phospho-specific antibodies. The changes in phosphorylation were similar in all three lines. To evaluate the specific downstream regulator of 5′cap binding, we used an antibody to 4E-BP1 and ran lysates on a high concentration (15%) acrylamide gel. This allowed us to observe changes in the hypophosphorylated active 4E-BP1 [456, 457]. In all three cell lines hypoxia increases the lowest molecular weight 4E-BP1 band.
Figure 2.11: Phosphorylation Status of Components of Pathways Regulating Translation Characterizes Their Activity in Hypoxia.

The mTOR/4E-BP1 pathway and the eIF2α pathway are active in response to hypoxia in T47D and MCF7 cancer cells similar to the response seen in non-transformed H9 human embryonic stem cells. T47D (a), and MCF7 (b) cancer cells were cultured for up to 24h in 0.5% oxygen. H9 (c) human embryonic stem cells were cultured at 1% oxygen along a 24h time course. Antibodies to phosphorylated mTOR (Ser2448, Ser441) were used to probe immunoblots. Total mTOR was used as a loading control. Immunoblot analysis of using phosphorylated eIF2α (Ser51) antibodies was also performed. Total eIF2α was used as a loading control. Phosphorylation of 4E-BP1, for each cell line is also shown in response to hypoxia by downward shifts in molecular weight. Representative blots for three independent experiments are shown (n=3). Cancer cell lines respond similarly to the non-transformed H9 HESCs cells.
Figure 2.12: Chemotherapy Induces a Similar Signalling Response to Hypoxia in Pathways that Govern Major Translational Responses to Stress.

In both (a) the T47D cell line and (b) the SUM149 cell line chemotherapy (paclitaxel 20nM) elicits an increase in the phosphorylation of eIF2α (Ser51) and a decrease in the phosphorylation of 4E-BP1 as measured by western blot probe with eIF2α-p (Ser51) antibody or 4E-BP1 antibody. Activation of 4E-BP1 is represented by the accumulation of hypophosphorylated protein accumulated in the lower 4E-BP1 bands. This response is similar to that seen in hypoxia. Both β-Actin and total eIF2α are provided as loading controls. Blots are representative of three independent experiments.
Figure 2.13 Diagram of Luciferase Reporters Vector and ATF4 and ATF4 Mutant Promoter.
a  HEK-293

Fold Change in Luciferase Activity (log₂)

<table>
<thead>
<tr>
<th>Condition</th>
<th>ATF4</th>
<th>Mut ATF4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>TG</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

b  MDA-MB-231

Fold Change in ATF4 Transcript Abundance Bound to Ribosomes (log₂)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Monosomes</th>
<th>Low MW Polysomes</th>
<th>High MW Polysomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>1</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>1</td>
<td>8</td>
<td>16</td>
</tr>
</tbody>
</table>

c  MCF7

Fold Change in ATF4 Transcript Abundance Bound to Ribosomes (log₂)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Monosomes</th>
<th>Low MW Polysomes</th>
<th>High MW Polysomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>1</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>1</td>
<td>8</td>
<td>4</td>
</tr>
</tbody>
</table>
Figure 2.14: Hypoxia Alters the Translational Efficiency of ATF4 mRNA as Controlled by Upstream Open Reading Frame (uORF).

(a) ATF4 5’UTR showed increases translational efficiency in hypoxia as measured by luciferase activity. The mutation of a uORF from ATG to ATA makes the 5’UTR less responsive to hypoxia-induced translational activation. Vectors containing ATF4 5’UTR or Mut ATF4 upstream of the firefly luciferase gene were transiently transfected into HEK-293 cells that were left untreated (normoxia), exposed to 0.5% O2 for 6 hours (hypoxia) or given 0.1 µM of thapsigargin (TG) for 3h (n=3). In both experimental conditions, hypoxia and TG, mutation of the ATF4 uORF decreased translational efficiency (p<0.05). (bc) Fold change in transcript abundance bound to one of three polysome profile fractions — monosomes, low molecular weight polysomes (Low MW Polysomes), or high molecular weight polysomes (High MW Polysomes). Both the MDA-MB-231 (b) and MCF7 (c) cell lines were tested. Real time RT-PCR demonstrated changes in ribosome occupancy as caused by low oxygen conditions (0.5% O2 for 24h) compared to normoxia (20% O2 for 24h). All values are normalized to total transcript for their respective conditions. The levels of ATF4 increased their association with high molecular weight polysomes indicating an increase in translational efficiency in both the MDA-MB-231 (n=5) and MCF7 (n=4) breast cancer cells. The bars show the mean fold change ± SD. Horizontal lines indicate a significant difference (p<0.05, n=3).
suggesting that 4E-BP1 is active and capable of inhibiting translation in response to hypoxia. eIF2α phosphorylation is the culminating step of multiple signalling pathways that prevent the formation of the ternary complex, and thus inhibit translation under conditions of stress. In all three cell lines we see an increase in the eIF2α phosphorylation required for increased eIF2B binding and ternary complex formation inhibition. In our models of breast cancer, the pathways that regulate translation are competent for signalling in response to hypoxia and signal similarly to non-transformed cells. It has been shown that HIF1α can mediate chemotherapeutic resistance through the upregulation of CSCs, therefore we hypothesized that translational stress responses may so too be co-opted to promote chemoresistance [458]. To determine if translational responses may be required for adaptation to chemotherapy, we evaluated the same pathways as in Figure 2.11 — 4E-BP1 and eIF2α — to see how they respond to 20nM paclitaxel. This response was tested in two different breast cancer cell lines T47D (Fig. 2.12 a) and SUM149 (Fig. 2.12 b). Similar responses were observed in each cell line; the phosphorylation of eIF2α increases and there is an accumulation of the hypophosphorylated 4E-BP1 band. As hypothesized the pathways are activated by chemotherapeutic stresses such as paclitaxel.

We were graciously supplied with two vectors from the Koromilas lab. Figure 2.13 is a schematic of the vectors. These are UTR luciferase reporter vectors into which the 5’UTR of a specific gene can be cloned downstream of the partial TK promoter and upstream of the firefly luciferase gene. The two vectors we received contained the ATF4 UTR and the ATF4 UTR with the first uORF mutated from AUG to AUA [403, 459] (Fig. 2.13 b). ATF4 is an important translationally activated downstream effector of the ISR that promotes pro-tumourigenic phenotypes like migration and metastasis [403, 409].

2.2.4 Translation Efficiency Promotes Pro-Survival Gene Expression Under Conditions of Translation Repression

We implemented two complementary methods to assess translational efficiency: luciferase bioluminescence assays and polysome profiling. To test the UTR efficiency using the luciferase assay we transfected HEK293 cells with either the ATF4-vector or mutant ATF4-vector (Mut ATF4) and treated the cells. Two stresses were tested. Cells
Figure 2.15: Figure 2.15: Hypoxia Increases the Translational Efficiency of VEGF-A mRNA and Decreases the Translational Efficiency of β-Actin mRNA (ACTB).

The quantity of mRNA bound to polysome profile fractions, normalized to total transcript levels as assessed by real time RT PCR and presented as fold change comparing normoxia to hypoxia. (a) In MDA-MB-231 cells VEGF-A increases its association with high molecular weight polysomes (High MW Polysomes, n=4) when exposed to 0.5% O2. Conversely, β-ACTIN is found primarily on the fractions associated with less efficient translation, monosomes and low molecular weight polysomes (High MW Polysomes). (b) VEGF-A and β-ACTIN increase and decrease their association with high molecular weight polysomes respectively following hypoxia treatment in MCF7 cells (n=4). Values were first normalized to the mRNA levels from unfractionated RNA extracts from the same conditions, then normalized as fold change to normoxia monosomes. The bars signify the mean fold change ± SD. Horizontal lines indicate a significant difference as determined by Students t-test (p<0.05).
Fold Change in OCT4 Transcript Abundance Bound to Ribosomes (log2)

**a** MDA-MB-231

- **b** SLUG (SNAI2)
- **c** SOX2
- **d** TWIST1
- **e** GAPDH
Figure 2.16: Drivers of Glucose Metabolism, EMT and Pluripotency Show Resistance to Hypoxic Down-Regulation of Transitional Efficiency in MDA-MB-231 cells.

Monosomes, low MW polysomes, and high MW polysomes were collected and analyzed using real time RT PCR for mRNAs associated with EMT, glycolysis, or plasticity. (b) Slug (SNAI2) (p= 0.0022), (d) Twist (TWIST1) (p=0.0007) and (e) GAPDH (p=0.04) mRNA showed increased ribosome binding in multiple fractions in hypoxia, where pluripotency factors, (a) OCT4 (p=0.2266) and (c) SOX2 (p=0.0817), showed neither a significant increase, nor a decrease in their association with high molecular weight polysomes. All samples were normalized first to the total amount of a given transcript then normalized to normoxia monosomes. Each mRNA was tested from a minimum of three independent experiments (OCT4 n=4). The bars signify the mean fold change ± SD. Horizontal lines indicate a significant difference calculated using Student’s t-tests (p<0.05).
were exposed to a 6h hypoxia insult or to 0.1 µM thapsigargin (TG), a known inducer of ER stress and ATF4 translational expression, which was used as a positive control (Fig. 2.14 a) [460]. Under both stress conditions the intact 5’UTR of ATF increases luciferase activity more than that of the uORF1 mutated ATF4 5’UTR (Mut ATF4). Next, we tested whether endogenous ATF4 showed differential translational efficiencies in hypoxia compared to normoxia. To do this cell lysates from cycloheximide treated cells were fractionated by sucrose gradient, then the absorbance of the gradients was measured, and samples were collected and pooled into three fractions: monosomes, low molecular weight polysomes (Low MW), and high molecular weight polysomes (High MW) (see Supplemental Figure 1 for pooled fractions). RNA was extracted from these fractions and measured using real time RT-PCR for ATF4. To control for transcriptional changes in overall ATF4 expression, all values were normalized to total ATF4 for their respective condition. In aggressive cancer cells, MDA-MB-231 cells (Fig. 2.14 b), and the less tumourigenic MCF7 cells (Fig. 2.14 c), ATF4 shows greater association with ribosomes in both the Low MW and High MW fractions when the cells are exposed to hypoxia (0.5% O₂) for 24h than in normoxia (20% O₂). With these data we have verified that the ATF4’s 5’UTR can translationally upregulate gene expression in hypoxia in a uORF dependent manner and that in breast cancer cell lines ATF4 is translated more efficiently in hypoxia than in normoxia.

To follow the analysis of ATF4, an ISR protein, we tested two other transcripts with known translational responses to hypoxia: VEGF-A and ACTB (β-ACTIN). VEGF-A is translationally upregulated in hypoxia by a putative IRES and β-ACTIN mRNA has been shown to be poorly translated but is not a 5’TOP mRNA [381, 461]. The same fractionation and pooling techniques were utilized in these experiments as were employed in the previous ATF4 experiment (Fig. 12.14). mRNA isolated from each of the fractions was analyzed and normalized to the total mRNA for each condition. In MDA-MB-231 cells VEGF increased its association with all three ribosome fractions. This increase could be observed even after accounting for the transcriptional increase in VEGF-A (p<0.05, n=4). Conversely, β-ACTIN showed a dramatic decrease in association with the most efficiently translating polysomes in the high MW fraction (Fig. 2.15 a).
### Monosomes

**Fold Change in OCT4 Transcript Abundance Bound to Ribosomes (log2)**

- **Normoxia**: Decreased
- **Hypoxia**: Increased

### Low MW Polysomes

**Fold Change in OCT4 Transcript Abundance Bound to Ribosomes (log2)**

- **Normoxia**: Increased
- **Hypoxia**: Increased

### High MW Polysomes

**Fold Change in OCT4 Transcript Abundance Bound to Ribosomes (log2)**

- **Normoxia**: Increased
- **Hypoxia**: Increased

---

### Normoxia vs. Hypoxia

- **OCT4**
  - Increase in polysomes
- **SNAIL (SNAI1)**
  - Increase in polysomes
- **SOX2**
  - Increase in polysomes

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### OCT4

- **Normoxia**: Decreased
- **Hypoxia**: Increased

### SNAIL (SNAI1)

- **Normoxia**: Decreased
- **Hypoxia**: Increased

### SOX2

- **Normoxia**: Decreased
- **Hypoxia**: Increased

---

### GAPDH

- **Normoxia**: Decreased
- **Hypoxia**: Increased
Figure 2.17: Drivers of Glucose Metabolism, EMT and Pluripotency Show Resistance to Hypoxic Down-regulation of Translational Efficiency in MCF7 cells.

Extracted mRNA from MCF7 monosomes, low MW polysomes, and high MW polysomes were assayed using real time RT PCR for (a) OCT4, (b) SNAIL (SNAI1), (c) SOX2,(d) GAPDH mRNAs. All samples were normalized to mRNA from whole cell extracts for their corresponding condition—20% O2, or 0.5% O2. No tested transcripts were shown to decrease their translational efficiency in response to hypoxia as measured by binding to high MW polysomes (0.5% O2 for 24h). Each mRNA was tested from three independent experiments. The bars signify the mean fold change ± SD and the horizontal bar indicates a Student’s t-test p<0.05.
(p<0.05, n=4). In MCF7 cells the increase in VEGF-A translational efficiency was even more profound with the association between mRNA and both polysome fractions increasing approximately 16-fold. β-ACTIN mRNA decreased its association with the high MW polysomes (Fig. 2.15 b) (p<0.05, n=4). The differential association of ATF4, VEGF-A, and β-ACTIN establish that our method can distinguishing the mRNA that escape the global downregulation of translation observed in Figure 2.9 a, b. Consequent to confirming the role translation plays in driving the expression of hypoxia-regulated proteins that govern phenotypes like stress response and vascularization, we sought to evaluate if factors enhancing plasticity also escaped translational repression. Pooled fractions, like those used in previous experiments from normoxia or hypoxia, treated MDA-MB-231 cells were tested for OCT4 (Fig. 2.16 a), SLUG (SNAI2) (Fig. 2.16 b), SOX2 (Fig. 2.16 c), TWIST1 (Fig. 2.16 d) and GAPDH (Fig. 2.16 e). SNAI2, TWIST1, and GAPDH all demonstrated a heightened affinity for ribosome binding in hypoxia resulting in an increase in mRNA in both polysome fractions (p<0.05, n=4). The pluripotency factors seem to react differently than any of the previously investigated transcripts. Where ATF4, VEGF-A, GAPDH, SLUG, and TWIST1 increased translational efficiency resulting in more ribosomes bound per transcript, and β-Actin mRNA decreased in its ability to initiate and maintain translation, OCT4 and SOX2 both maintained their translational efficiency. This is effectively an escape from the global decrease in translation, but has different dynamics compared to any other mRNA we have observed. Whether the transcripts fall into the category of ‘translationally enhanced’ or ‘translationally maintained’ these data suggest that mRNA that promote plasticity have continued access to translational machinery in times of stress.

We analyzed MCF7 cells (Fig. 2.17 a) using the same methodology and normalization as the MDA-MB-231 cells (Fig. 2.16). Neither SLUG nor TWIST1 was consistently measurable bound to polysomes, however SNAIL(SNAI1) another EMT transcription factor was (Fig. 2.17 b). All three of the transcripts, OCT4 (Fig. 2.17 a), SNAI1 (Fig. 2.17 b), SOX2 (Fig. 2.17 c), were ‘translationally maintained’ in the high MW fractions, OCT4 was increased by hypoxia in the low MW polysomes, and SNAIL was significantly
decreased by hypoxia in the monosome pool. GAPDH was increased in all fractions (Fig. 2.17 d). All samples were normalized to the total of their specific mRNA to account for any transcriptional influence. It may be that, like with many other forms of adaptation, less aggressive and more differentiated cancer lines may be less capable of utilizing translational adaptation compared to less differentiated cell lines.

2.2.5 Features of the 5’UTR Regulate Translational Efficiency

Analyzing the CAGE TSS HMM Encode/Riken data set we discovered that many of the genes regulating plasticity had multiple unique RNA sequences detectable upstream of the start codon indicating the presence of multiple 5’UTRs. In some cases, this would result in multiple transcripts encoding the same protein but with different 5’UTRs. Broadly, there were three types of 5’UTRs. There were those unique 5’UTR that resulted from different TSSs. These can be identified by either the presence or absence of RNA sequence at the 5’ most end of the transcript. There were those 5’UTR that derived from alternative splicing within, which are characterized by the presence or absence of an exon sequence in the middle of the UTR. The final alteration that we observed was the identification of a different start site other than the primary start site. This type of modification has the added complication of not only changing the UTR sequence but simultaneously altering the protein sequence. Expanding this complexity, some 5’UTRs are consistently attributed to a single splice variant while other splice variants of the same gene may have several different 5’UTRs. For our subsequent investigation we limited our experiments to different 5’UTR that are likely associated with transcripts containing the same coding region. Though this is obviously less comprehensive than exploring more 5’UTRs, this option eliminates other cis-factors as confounding variables.

From the Riken data set we identified three different NODAL variant-5’UTRs and detected a 4th using 5’ Rapid amplification of cDNA ends (RACE) (Fig. 2.18 a). We designed primers to detect NODAL 416 (NODAL_416_amplicon) and NODAL 298 (NODAL_298_amplicon), which contained substantial amounts of unique sequence. NODAL 42, the canonical NODAL 5’UTR, lacked a unique sequence so it is detected simultaneously with 298 (NODAL_small_amplicon). NODAL 14 suffers from a similar
b  MDA-MB-231

Fold Change in Transcript Abundance Bound to Ribosomes (log_2)

- Monosomes (N)
- Low MW Polysomes (N)
- High MW Polysomes (N)
- Monosomes (H)
- Low MW Polysomes (H)
- High MW Polysomes (H)

c  MCF7

Fold Change in Transcript Abundance Bound to Ribosomes (log_2)

- Monosomes (N)
- Low MW Polysomes (N)
- High MW Polysomes (N)
- Monosomes (H)
- Low MW Polysomes (H)
- High MW Polysomes (H)
Figure 2.18: NODAL 5’UTR Utilization Changes in Hypoxia.

(a) Target 5’UTR were chosen using the UCSC genome browser identifying both annotated and putative 5’UTR from the available CAGE data. These 5’UTR were then verified by real time RT PCR. (b) Real time RT PCR for different 5’UTR expressed by MDA-MB-231 cells demonstrates that some 5’UTR are preferentially utilized under hypoxic conditions at 24h —416 and a UTR detected by the Nodal Small primers (p<0.01). Two 5’UTRs were detected directly, Nodal 416 and Nodal 298, along with a set of 5’UTRs detected by the Nodal Small primers, including Nodal 42 and 298 (n=3). Each 5’UTR was normalized to the total amount found in whole cell extract.  (c) MCF7 cells exposed to hypoxia, analysed in the same way as the MDA-MB-231 cells (b) but did not show a significant increase in the efficiency of any of the NODAL 5’UTR in hypoxia (n=4). 5’UTR did show very different efficiencies when compared to one another in hypoxia. The bars show the mean fold change ± SD. Horizontal lines indicate a significant difference as calculated by Student’s t-tests (p<0.05).
lack of unique sequence exacerbated by the sequence being common to all NODAL 5’UTR and thus cannot be meaningfully examined by PCR. As most of these UTRs have never been reported, it was important to determine if they were translationally active to help rule out other cellular functions like those of non-coding regulatory RNAs. We were able to detect the NODAL 416, NODAL 298, and the sequence for NODAL Small (298 and 42) on polysomes demonstrating that translation is initiated from these 5’UTR (Fig. 2.18 b, c). Distribution throughout the profiles suggest these mRNAs are fully competent in their capacity to facilitate initiation and elongation. Furthermore, by analyzing the monosome, low MW, and high MW polysome fractions from cells grown in normoxic (20% O₂; N) or hypoxic conditions (0.5% O₂; H), we can identify if the NODAL 5’UTRs have different translational efficiencies, and if any of the 5’UTRs are differentially regulated by hypoxia. In figure 2.18 b the NODAL 416 UTR shows higher association with both polysome fractions, low MW (N) and high MW (N), than the other 5’UTRs. This higher baseline translational efficiency can be found in the MDA-MB-231 cells but not in the MCF7 cells. We also show that, in MDA-MB-231 cells the 5’UTRs have different kinetics. The NODAL 298 5’UTR shows no translational response to hypoxia in a fraction, which is different from NODAL 416 and NODAL Small which both show an increase in their association with high MW, efficiently translating, polysomes. Even among these two hypoxia responsive transcripts we observe different dynamics (Fig. 2.18 b) (p<0.01, n=4). NODAL 416 increases affinity for polysomes to a greater extent than the observed increase in NODAL Small, but inferences from these data are limited because NODAL Small does not represent a distinct transcript (Fig. 2.18 b). In the less aggressive MCF7 line we see a muted NODAL translational response. Only the NODAL 416 5’UTR shows improved translational efficiency compared to the other 5’UTR. Among the polysome samples low MW (N), high MW (N), low MW (H), and high MW (H), only the high MW (H) NODAL 416 showed significant changes from the rest of the groups. In MCF7 cells NODAL 416 was more efficiently translated than the other 5’UTRs and it was the only NODAL 5’UTR that showed a significant increase in translational efficiency when cells were exposed to hypoxia (Fig. 2.18 c) (p<0.05, n=4).
**Mean Fold Change in Luciferase Activity (log2)**

**Normoxia**  
**Hypoxia**

**NODAL 14**

**Mean Fold Change in Luciferase Activity (log2)**

**NODAL 42**

**Mean Fold Change in Luciferase Activity (log2)**

**NODAL 298**

**Mean Fold Change in Luciferase Activity (log2)**

**NODAL 416**

**Mean Fold Change in Luciferase Activity (log2)**

**Fold Change in Luciferase Activity (log2)**

**Normoxia**  
**Hypoxia**

**NODAL 298**

**Mut298**

**Coding Sequence**

**NODAL 298 5’UTR**

**Initiation**

**Coding Sequence**

**Initiation**

**Luciferase**

**Luciferase**
Figure 2.19: Luciferase Activity of NODAL 5′ UTR Reporters in Hypoxia.

(a) Four NODAL 5′ UTRs were cloned and inserted upstream of the firefly luciferase gene. HEK-293 cells were transiently transfected with one of the 5′ UTR-containing vectors and were then cultured in normoxic or hypoxic conditions for 6h (20% O2, or 0.5% O2). NODAL 42, the annotated NODAL 5′ UTR, along with NODAL 298 and 416 showed a significant increase in luciferase activity. The bars signify the mean fold change in luciferase activity ± SD. Asterixis (*) indicate a significant difference as calculated by Student’s t-tests (p<0.05, n=4). (b) NODAL 298 and NODAL Mut298 (AUG to AUA mutation) 5′ UTRs were cloned and inserted upstream of the firefly luciferase gene. HEK-293 cells were transiently transfected with one of the 5′ UTR-containing vectors and were then cultured in normoxic or hypoxic conditions for 6h (20% O2, or 0.5% O2). The uORF in the NODAL 298 is required for efficient translation (p<0.01, n=3). The bars signify the mean fold change in luciferase activity ± SD. Letters indicate a significant difference as calculated by ANOVA (p<0.05, n=4). (c) Diagram of the NODAL 298 and NODAL Mut298 luciferase reporters.
Figure 2.20 Luciferase Reporter Activity of Unique 5’UTRs from Pluripotency genes in Hypoxia.

NANOG 290, NANOG 350 (a), OCT4 413, and OCT4 961(c) were each cloned upstream of the partial TK promoter and firefly luciferase gene. Each reporter vector was transfected into HEK-293 cells, which were in turn cultured in either 20% O2 or 0.5% O2 for 6h. All experiments were completed four times. The canonical NANOG 5’UTR 350 (p=0.048) and both OCT4 5’UTR, 413 (p=0.031) and 961 (p=0.022) displayed increased translation in response to low O2 stress (n=4). The bars signify the mean fold change ± SD.
Polysome profiling is an effective method of determining translational efficiency in a specific cell type, but as previously discussed expression of eIFs, or sequences in the coding regions or 3’ UTRs may also affect translation. To overcome these potentially confounding variables and to test the 5’UTRs, which could not be detected with PCR, we evaluated the regulatory role of the four NODAL 5’UTRs using the previously described luciferase reporter system (Fig 2.13). Without the contributions from sequences in the coding region or 3’ UTR we can better identify how the 5’UTRs react to hypoxic stress. To do this, synthetic oligonucleotides of each of the four 5’UTR were cloned into the luciferase vector. These vectors were cloned into HEK293 cells and treated in hypoxia seemed capable of facilitating some increase in translation to hypoxia in HEK293 (0.5% O₂) for 6 hours. The cells were lysed, and the luciferase activity measured. Though all four of the tested NODAL 5’UTR — NODAL 14, 42, 298, 416 (Fig. 2.19 a) — cells canonical NODAL 42 as well as the two long NODAL 5’UTR — 298, 416 — reached significance (p<0.05, n=4) (Fig. 2.19). NODAL 298 or the AUG to AUA NODAL 298 mutant (Mut298) reporter vector were transfected into HEK293 cells. The absence of the uORF reduces the efficiency of translation under normal condition and mitigates the upregulation in response to hypoxia (Fig. 2.19 b).

As our data and the data of many others suggest that NODAL is not the sole driver of hypoxia-induced plasticity, we evaluated the 5’UTR of other pluripotency factors, specifically, NANO G (2.20 a, b) and OCT4 (2.20 c, d). For the NANO G 5’UTR we used the canonical 5’UTR, Nanog 219, and a second that was reported in Eberle et al., Nanog 350 [462]. The existence of both 5’UTRs is confirmed in the Riken Nano Cage data set. Using the same methodology from Figure 2.19 the NANO G 5’UTRs were cloned and transfected into HEK293 cells. Nanog 219 shows a statistically significant increase in luciferase activity in response to 6h of hypoxia (p=0.048, n=4) (2.20 a, b). Employing the luciferase reporter system, we investigated two OCT4 5’UTRs, OCT4 413 and OCT4 961. The OCT4 413 seems to be produced from the exclusion of an exon in the middle of the 5’UTR, though both 5’UTRs have the same TSS. Both OCT4 5’UTRs can facilitate an increase in translation in response to hypoxia and many of the NODAL and OCT4, NANO G mRNA 5’UTRs are capable of increasing or maintaining translation under
Figure 2.21: The Transcription Factor SNAIL’s 5’ UTRs Translate with Different Efficiencies Under Stress Conditions.

(a) Diagram of SNAIL’s two 5’UTRs and amplicons produced in their detection by real time RT PCR. (b) 5’UTR luciferase reporters were generated for the 417 and 85 5’UTR. (b) Luciferase reporters were transiently expressed in HEK-293 cells, which were either left untreated (normoxia), exposed to 0.5% O2 for 6 hours (hypoxia) or 0.1 µM of thapsigargin for 3h (n=3). The proximal promoter facilitated increased translation in response to either stress to a greater extent than the distal promoter. The bars present the mean fold change ± SD, and horizontal lines indicate p<0.05.
Figure 2.22: SNAIL’s Proximal (SNAIL 85) 5’UTR Results in Higher Ribosome Occupancy in Hypoxia.

In hypoxic conditions MCF7 (a) and MDA-MB-231 (b) breast cancer cell lines the SNAIL proximal promoter is found more commonly bound to ribosomes in both the low MW and high MW polysome fractions. Shown are the fold changes in transcript abundance bound to ribosomes in one of three fractions of the polysome profiles monosomes, low molecular weight polysomes, or high molecular weight polysomes. Real time RT-PCR demonstrated changes in ribosome occupancy as caused by low oxygen conditions (0.5% O2 for 24h) compared to normoxic conditions (20% O2 for 24h). All values are normalized to total transcript for their respective conditions. All experiments were completed in triplicate. The bars show the mean fold change ± SD. Horizontal lines indicate a significant difference (p<0.05, n=3).
conditions of hypoxia. The data in these cancer lines suggests that yet unidentified mechanisms impart further regulation upon these mRNAs.

SNAIL, a key driver of EMT in breast cancer, is associated with CSC formation and is regulated by hypoxia [183, 463-465]. We also found that SNAIL has two discreet 5’ UTRs, which we have termed SNAIL 417 and SNAIL 85, that differ by more than 300bp (Fig. 2.21 a). In our luciferase reporter assays in HEK293 cells, both 5’ UTRs upregulated the expression of luciferase in response to 6h of hypoxia treatment and SNAIL 85 significantly upregulated luciferase activity with a 3h exposure to TG (Fig. 2.21 b) (p<0.05, n=3). Under both stress conditions the small SNAIL 5’ UTR, SNAIL 85 was more responsive to stress than the longer SNAIL 417. Though it seems that these 5’ UTRs are capable of enabling escape from hypoxia-induced translational downregulation of gene expression we have to verify the function of these 5’ UTRs within the specific cellular context of breast cancer.

In MCF7 cells both 5’ UTRs were detectable though SNAIL 85 was more abundant and is translated more efficiently under normoxic conditions than SNAIL 417. Translational efficiency is measured as greater association with the rapidly translating high MW polysomes (Fig 2.22 a). When the MCF7 cells are exposed to 0.5% O2 for 24h the relative differences in the association with polysomes between these two mRNAs increases, making the differences between SNAIL 85 and SNAIL 417 significantly different in low (p=0.008) and high (p=0.009) MW polysomes. SNAIL 85 maintains its full translational efficiency in hypoxia, but SNAIL 417 decreases its association with HW polysomes relative to its association in normoxia (Fig 2.22 a) (p<0.05, n=3). The MDA-MB-231 cells show very similar responses (Fig. 2.22b). SNAIL 85 maintains its translational efficiency under hypoxic stress. Under conditions of hypoxia SNAIL 85 containing mRNA show greater association with monosomes and both polysome fractions than those with the SNAIL 417 5’ UTR (p<0.05 n=3). This difference was not present under the steady state normoxic
conditions. The other notable observation is that in MDA-MB-231 cells the SNAIL 417 UTR containing mRNA decreased its association with all three fractions in hypoxia relative to their respective normoxic fractions (p<0.05 n=3). These two SNAIL 5′UTRs give us the clearest view yet of how the synthesis of proteins that control cell state and plasticity can be regulated by the differential expression of 5′UTRs.

2.3 Discussion

The first major goal of this chapter is to establish the effects of stress on cancer stem cells and plasticity. To do this we focus on hypoxia as an endogenous stress that promotes cancer progression. We identify translation in stress that acts as a central regulatory node from which phenotypes like survival, plasticity, and metabolism are simultaneously regulated. Other groups have identified other translationally regulated processes like metabolism, HIF1α-mediated hypoxic responses, and vascularization that also possesses a translational component [466-468]. We also begin to explore if other stresses like chemotherapy may elicit similar responses. Using polysome profiling we were able to identify many important regulators of plasticity that escape hypoxia-induced downregulation. Finally, from these data we establish that transcripts of the same gene often contain different 5′UTRs that confer different translational efficiencies especially in times of stress. Most of these are previously unknown 5′UTRs.

Evidence continues to accumulate that many different stresses can induce CSC formation, EMT, or plasticity [469-471]. In our RNAseq analysis of hypoxia treated T47D cells EMT emerged as the third most upregulated set of pathways by GSEA. To better focus our investigation, we primarily investigated EMT markers, as suggested by the RNAseq data, and stem cell genes as we have previously identified NODAL, which belongs to a regulatory network that includes NANOG and OCT4, as a feature of hypoxic adaptation in breast cancer cells [472]. EMT and the stem cell signature are well characterized as generating plastic phenotypes in breast cancer [189, 192]. We identify a number of genes that are potential contributors to the acquisition of plasticity in response to hypoxia. In most of the breast cancer lines we tested, we identified increases in the mRNA expression of genes associated with plasticity. Of the transcripts tested OCT4 was the most commonly upregulated by hypoxia in our cell lines. These changes in gene expression
occur concurrently with increases in CSCs as measured by sphere formation and flow cytometry (Fig 2.1 and Fig. 2.4 b). Though we use flow cytometry to identify CSCs, our primary output in most experiments is sphere formation as these experiments have been shown to correlate well with xenograft transplantation assays and are being used as secondary outcomes in clinical trials [68, 447-449, 473, 474]. Having previously established NODAL’s upregulation in response to hypoxia, we sought to determine if NODAL increased CSCs. The overexpression of NODAL or the administration of rhNODAL increase CSC formation. Though the effects of rhNODAL were significant, the magnitude of the effect was much smaller than that of hypoxia, suggesting that NODAL is only one part of the hypoxia-induced plasticity network required for CSC formation. Furthermore, in vivo where hypoxia occurs naturally due to the oxygen diffusion gradients that result from the structure of the tumour and tumour vasculature, we find that the cells expressing higher levels of the hypoxia marker CA9 possess greater levels of NODAL expression. This co-expression of CA9 and NODAL was found in SUM149 tumours, as well as those tumours derived from two different PDX lines. These data show that hypoxic stress increases phenotypic plasticity and CSC formation by promoting the expression of proteins like NODAL. Plasticity and CSCs have been repeatedly shown to be drivers of chemoresistance and metastasis (reviewed in [475] and [476]). It follows then that inhibiting the adaptive response to hypoxia may cause the attenuation of multiple mechanisms that promote neoplastic progression and tumour survival.

There have been many groups that have shown promising results targeting HIF1α and CA9 [477-480]. Limiting the adaptive response to hypoxia could potentially cause greater apoptosis and limit the effects of clonal selection, prevent the acquisition of plasticity, reduce therapy resistance, decrease CSC formation, and limit vascularization. All of these ideas are supported individually by the literature, but it has yet to be shown if targeting the hypoxic stress responses is sufficient to reduce them enough to improve the survival of patients [272, 477-480].

A unique feature of NODAL’s expression in hypoxia may provide evidence for a novel mechanism to target stress responses in breast cancer. We previously demonstrated, and
in this chapter more conclusively confirmed, that NODAL is not solely regulated at the level of transcription in hypoxia (Fig. 2.8). We have hypothesized two mechanisms by which this can occur; the first is protein stability (Fig. 2.7) and the second was selective translation. In hypoxia NODAL is more stable than it is in normoxia, accounting for some of the increase in protein expression observed. We confirmed that in our models hypoxia decreases global translation by 70%-80% (Fig. 2.9). In times of stress the proteins required for survival and adaptation must escape this reduction in translation. There are two well-characterized pathways that regulate the initiation of translation mTOR/4E-BP1 and PERK/eIF2α. We demonstrate that these pathways are active in breast cancer where they signal similarly to non-transformed cells indicating that the signalling has not been disrupted by mutation or transformation. We observe an increase in active 4E-BP1, which is known to decrease cap-binding and an increase in eIF2α-p, which prevents ternary complex recycling. These translational responses are required for survival, treatment resistance and the maintenance of EMT [410, 481, 482]. Though hypoxia is an important stress that acts throughout the natural history of the tumour to promote cancer progression, it is only one of the many stresses tumours endure. We tested to see if chemotherapeutic stress induced by paclitaxel resulted in the similar activation of pathways regulating translational responses. We observe the accumulation of the hypophosphorylated 4E-BP1 and the phosphorylation of eIF2α. These results mirror the results we observe in hypoxia.

As all protein expression is ultimately dependent on translation, and cells respond to certain stresses by downregulating translation to conserve energy and protect the cell two potential strategies to exploit this for therapeutic benefit emerge. The first is to add a second stressor to further activate the same pathway driving the cells towards cell death [410, 483]. The second would be to prevent the adaptive response and let the cells succumb to the initial stress, be it hypoxia or chemotherapy. This second concept will be explored in the following chapters. It is likely that both of these are viable options and likely depend on the extent to which the stress pathways are activated in the initial tumour. Conceptually, there is a concern with adding a second stressor as part of cancer therapy. Adding a stressor, like an activator of the UPR, may have similar results to the current cytotoxic therapies that seek to stress and ultimately kill cancer cells, whose side
effects are caused by stressing or damaging non-tumourigenic cells. Inhibiting a stress pathway to prevent adaptation would likely produce a multitude of other cellular stresses, but they would be confined to those cells that required the pathway being inhibited, namely the cells suffering an environmental insult. Though both these methods have potential clinical benefits we believe there is a strong clinical rationale for choosing to inhibit stress responses.

We wanted to evaluate the role that translation plays in hypoxia-induced plasticity. To do this we used two methods to evaluate translational efficiency: quantifying mRNAs in association with polysomes and measuring luciferase activity in reporter assays. Through polysome profiling we were able to test the changes in translational efficiency in hypoxia in our cancer cell lines. With our luciferase assays we compared the different 5′ UTRs in a consistent genetic background independent of other regulatory elements found throughout the mRNA. This absence of confounding variables is both the strength and limitation of the assay. ATF4 is an important marker for the activation of the ISR and one of only a few genes with a well-characterized 5′ UTR translational regulation. We used ATF4 to verify the translational activation of the ISR and as a positive control demonstrating that our systems were adequately sensitive to detect translational changes in hypoxia. Here we replicate the result that ATF4 increases its translational efficiency in hypoxia in a uORF dependent manner. We characterize two other mRNAs that represent different translational responses to hypoxia; VEGF-A expression is dependent on both transcriptional and translational mechanisms and its expression is mediated by an IRES dependent mechanism; and β-ACTIN, a non-TOP mRNA, that has been found to lose translational efficiency under hypoxic conditions (Fig. 2.15) [381, 461]. We confirm both these effects in our cell lines.

Having measured the translational efficiency of well characterized regulators of stress response and vascularization, phenotypes consistently found to be controlled by hypoxia, we then examined the translational efficiency of plasticity promoting genes OCT4 and SOX2, part of the stem cell gene signature, and the EMT transcription factors SLUG, SNAIL or TWIST1 depending on the expression in the cell lines tested. Though the cell lines differ in their exact translational patterns, SLUG, TWIST1 and OCT4 show some
cell line specific ability to increase translational efficiency measured as the affinity of ribosomes to those transcripts in polysome profiles. All of the plasticity related mRNAs tested showed an ability to resist the global downregulation of translation. Though there are many mechanisms by which mRNAs are differentially regulated it appears that most of the stem cell and EMT transcripts we had investigated possessed multiple unique 5'UTRs. *SLUG* was a notable exception to this observation. We hypothesized that these different 5'UTRs would exhibit different translational activities and could be used to modulate gene expression in times of stress. It is also possible that tissue or cell state-specific expression of 5'UTRs may be important in differentiation. Of the *SNAIL* mRNAs we investigated it is notable, though not definitive, that the cancer lines use the 5'UTR that is common in H1 hESCs and not the one common in adult cells of the mesenchymal lineage, BJ human foreskin fibroblasts (Supplemental Figure 2). We also showed that when removed from other trans-acting factors, many of the 5'UTR can be permissive to translation in hypoxia, but this is not always mirrored in the cancer cells. Though there is additional work needed to further our understanding of how and why different 5'UTRs are used, and what sequence and what eIFs are required to alter translation, it is becoming clearer that escaping translational repression to facilitate gene expression is a necessary and hopefully targetable step in the process of gene expression.
2.4 Methods

2.4.1 Cell Culture and Treatments

T47D cells, obtained from ATCC (Manassas, Virginia, USA), were maintained in RPMI-1640 Medium (Life Technologies; Carlsbad, California, USA) with 10% fetal bovine serum (FBS) (Life Technologies) at 37°C with 5% CO₂. Cells were passaged using 0.25% (w/v) trypsin (Life Technologies) as per ATCC recommendations. MCF7 cells, obtained from ATCC, were maintained in RPMI-1640 Medium with 10% FBS at 37°C with 5% CO₂. Cells were passaged using 0.25% (w/v) trypsin as per ATCC recommendations. SUM149 cells, purchased from BioreclamationIVT, were grown in Ham's F-12 medium with 5% heat-inactivated FBS, 10mM HEPES, 1µg/mL hydrocortisone, and 5µg/mL insulin. All cells were grown in a humidified environment at 37°C with 5% CO₂.

H9 hESCs from WiCell (Madison, Wisconsin, USA) grown on irradiated CF-1 Mouse Embryonic Fibroblasts (GlobalStem; Gaithersburg, Maryland, USA) in knockout DMEM/F12 (Life Technologies; Carlsbad, California, USA), 20% knockout serum replacement (Life Technologies), 1X non-essential amino acids (Life Technologies), 2mM glutamine (Life Technologies), 0.1mM 2-mercaptoethanol (BME; Thermo Fisher Scientific; Waltham, Massachusetts, USA), and 4ng/mL of basic fibroblast growth factor (FGF) (Life Technologies). For experiments cells were passaged into feeder-free conditions. Feeder free conditions consisted of Geltrex matrix (Life Technologies) as a growth substrate and mTeSR1 media (Stem Cell Technologies; Vancouver, British Columbia, Canada). Paclitaxel (Pac; 20nM) (Sigma-Aldrich; St. Louis, Missouri, USA) was used as a chemotherapeutic stress. We employed recombinant human NODAL (R&D Systems, Minneapolis, Minnesota, United States) and SB431542 (10µM) (Sigma-Aldrich) to activated and inhibit NODAL signalling.

2.4.2 Hypoxia Treatments

Hypoxia was administered at the noted concentrations using Xvivo system (BioSpherix; Parish, New York, USA). The Xvivo’s continuous monitoring was used to ensure
consistent and accurate oxygen levels were maintained. Though different oxygen concentrations were used for different experiments, the temperature and CO\textsubscript{2} were consistent at 37\textdegree{C} with 5\% respectively. Upon the completion of the hypoxia treatment, cells were removed and processed for downstream application.

2.4.3 Sphere Formation

Sphere formation media was composed of DMEM/F12 + GlutaMax (Life Technologies), 1x B27 (Life Technologies), 20 ng/mL epidermal growth factor (EGF) (Life Technologies), and 10 ng/mL FGF (Life Technologies). After treatment, cells were harvested using 0.25\% (w/v) trypsin (Life Technologies), the trypsin was neutralized, and the cells resuspended in fresh media. These cells were filtered through a 40\textmu{m} pore filter (Thermo Fisher) to obtain a single cell solution. Cells were counted using trypan blue (Thermo Fisher) and diluted in the sphere formation media to the appropriate concentration for plating. Seed 200\mu{L} of the diluted cells into each well of a 96 well Ultra-Low Attachment Surface plates (Corning, NY, USA). Spheres were given between 10 and 21 days to grow.

2.4.4 Imaging Spheres

Images of spheres were captured using the EVOS FL Cell Imaging System (Thermo Fisher) at 4X magnification. Minimal changes were made to contrast and brightness to improve the visibility of the spheres.

2.4.5 RNA Extraction

The PerfectPure RNA Cultured Cell Kit (5-Prime; Hilden, Germany) was used to extract total RNA from cultured cells following the manufacture’s protocol using the following optional methods. Lysis buffer was added directly to the plate (option c for cell lysis manufacturer’s instructions). Optional DNase treatment was performed, and RNA was eluted in 50\mu{L}. Three (3) \mu{L} of purified RNA was used for quantification using the Epoch plate reader (Biotek; Winooski, Vermont, USA).
2.4.6 Reverse Transcriptase PCR and Complementary DNA Synthesis.

cDNA was made from purified total RNA using high capacity cDNA reverse transcription kit (Applied Biosystems; Foster City, California, USA) as per manufacturer’s protocol. The included random hexamers were used to prime reverse transcription and 1µg of RNA was used for each. ‘No Template,’ reactions without RNA and ‘No RT,’ a reaction without reverse transcriptase enzyme were included as controls.

2.4.7 Real Time PCR Analysis of Gene Expression

Real time PCR analysis was performed on 1µL of cDNA using TaqMan Gene Expression Master Mix according to the manufacturer’s procedures. FAM labelled TaqMan® gene expression human primer/probe sets (Thermo Scientific). mRNA expression was compared to untreated control using the ΔCT method. Data was collected on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad; Hercules, California, USA) using standard real time PCR settings.

1) Activation 95°C 10 min
2) Melting 95°C 15 sec
3) Annealing/ extension 55°C 1 min. Return to step 2 for 40 total cycles

Melt curve analysis was performed to ensure the production of a single amplicon.

The following 5’UTR specific primers were used: NANOG 350 forward primer GAT GGG GGA ATT CAG CTC AGG; NANOG 350 reverse primer TCA AGA CTA CTC CGT GCC CA; NANOG 291 forward primer AAC GTT CTG CTG GAC TGA GC; NANOG 291 reverse primer AGG CAG CTT TAA GAC TTT TCT GG; SNAIL 417 forward primer AAA GGG GCG TGG CAG ATA AG; SNAIL 417 reverse primer CGC CAA CTC CCT TAA GTA CTC C; SNAIL 85 forward primer CGG CCT AGC GAG TGG TTC; SNAIL 85 reverse primer CAC TGG GGT CGC CGA TTC; NODAL small (42 + 14 + 298) forward primer CTG GAG GTG CTG CTT TCA GG; NODAL small (42 + 14 + 298) reverse primer CAG GCG TGC AGA AGG AAG G; NODAL 298 forward primer GTT TGG TAC CTA GAG GAG CAG G; NODAL 298 reverse primer TCC AGG GAC GGG ATC TAG G; NODAL 416 forward primer CCC TCG GCA TTC TCT TCC TG; NODAL
416 reverse primer ATC CCT GCC CCA TCC TCT C. All other primer sets are included in Supplementary Table 1.

2.4.8 RNA-seq and Gene Set Enrichment

RNA was extracted from hypoxia treated cells with the Qiagen RNeasy kit and quantified via Nanodrop and quality was measured using Qubit. RNA was shipped to McGill University and Genome Quebec Innovation Center, where quality was validated via Bioanalyser, followed by NEB/KAPA library preparation and sequencing via Illumina HiSeq. Post sequencing quality check of reads was performed with FastQC and adapter sequences removed using Skewer. Reads were aligned to the GRCh38/Hg19 human reference genome using STAR. Data processing included Bigwig, PCA, correlation matrices, and coverage maps of aligned reads were produced using DeepTools. Read quantification via FeatureCounts was performed using Refseq annotations. Expression values for paired samples, hypoxia/normoxia, were obtained using the exact test within the edgeR package. An adjusted p-value (FDR) of 0.05 was used to determine statistically significant differences (p-value adjusted for multiple hypothesis testing by the Benjamini-Hochberg method). GAGE package for R was used to compare data to the hallmark gene sets from Molecular Signatures Database, which was used for Gene set analysis.

2.4.9 Plasmid Preparation

One Shot™ TOP10 Chemically Competent E. coli were transformed according to the manufacturer’s instructions and grown for 16h in liquid culture with the appropriate antibiotic for selection (50μg/mL kanamycin; (Life Technologies), 100μg/mL ampicillin; (Life Technologies)). Cells were pelleted at 3000g and purified using Geneaid Midi Plasmid Kit (Endotoxin Free) (Geneaid Biotech; Taipei City, Taiwan) as per manufacturer’s instructions.

2.4.10 Lipofectamine 2000 Transfection

Cell lines were transfected with plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufactures instructions. Stable cell lines were selected
with 500µg/mL until all sham transfected cell had died. Cells were maintained in 250µg/mL of G418).

2.4.11 Generation of the NODAL Overexpression Line

Lenti virural particles were purchased from GeneCopoeia (Montgomery County, Maryland).

- lenti-NODAL: NODAL Lentiviral Particles, Cat No. LPP-T9592-Lv105-050-S, titer 6.37x10^8 TU/ml
- lenti-GFP: eGFP Lentifect™ Lentiviral Particles, Cat No. LPP-EGFP-LV105-025, titer 1.26x10^8 TU/ml
- lenti-negative: Negative Control Lentifect™ Purified Lentiviral Particles, Cat No. LPP-NEG-Lv105-025, titer 1.1x10^8 TU/ml

Procedure of Transducing Sum149 cell:

Plate SUM149 cells (20 000 cells/well) into a 6-well plate. Replace the medium with 1 mL of medium containing polybrene at a concentration of 6 µg/mL. Thaw lenti-viral particles on ice, add LV-NODAL virus 4x10^6 TU (MOI=10) and 2x10^6 TU (MOI=5), LV-GFP virus 3.15x10^6 TU (MOI=11.2), LV-Neg 3.68x10^6 TU (MOI=9.2) into individual wells. Mix the medium by swirling the plate gently to ensure equal distribution of the virus. Incubate under stander culture conditions overnight. Remove the medium containing virus, replacing it with fresh complete culture medium allowing cells to recover overnight. On the following day remove the medium and replace it with fresh medium containing puromycin 500 ng/mL. Replenish selection medium every 3 days for 10 days.

2.4.12 Western Blots

Cells were lysed on-plate using Mammalian Protein Extraction Reagent (M-PER; Thermo Scientific), with Halt Protease Inhibitor Cocktail (Thermo Scientific), and Phosphatase Inhibitor (Thermo Scientific). Protein was quantified according to manufacturer’s instructions utilizing Pierce BCA Protein Assay Kit (Thermo Fisher) and measured on a FLUOstar Omega plate reader (BMG LABTECH; Offenburg, Germany).
4x Laemmli buffer (Bio-Rad; Hercules, California, USA) with 5% BME (Sigma-Aldrich; St. Louis, Missouri, USA) containing 20µg of protein was boiled for 10 minutes and loaded to be analyzed. Samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE), and then transferred onto Immobilon-FL membranes (Millipore). Membranes were blocked with 5% milk in PBS 0.1% Tween (Sigma-Aldrich) for 1h at room temperature, incubated with primary antibody overnight at 4°C (Supplementary Table 2). After the membranes were washed in PBS 0.1% Tween (Sigma-Aldrich) horseradish peroxidase (HRP)-conjugated secondary antibodies (Bio-Rad) were applied. The membranes were incubated for 1h at room temperature, washed to remove excess secondary antibody, and Clarity Western ECL Substrate (Bio-Rad) was used to detect signal. ChemiDoc™ XRS+ System (Bio-Rad) or film were used to image the western blots. Densitometry was performed using ChemiDoc™ XRS+ System (Bio-Rad).

Florescence western blot detection: Using the Trans Blot Turbo (settings of 25 V and 1.3 A for 15 minutes; Bio-rad) proteins were transferred to a low-auto-fluorescence PVDF membrane (Bio-rad), blocked for one hour at room temperature with Odyssey Blocking Buffer (Li-Cor; Lincoln, Nebraska, USA), then incubated with primary antibody overnight at 4°C in Odyssey Blocking Buffer with 0.1% Tween-20 (Sigma-Aldrich). Membranes were then probed with corresponding Li-Cor anti-mouse or anti-rabbit fluorescent secondary antibodies for one hour at room temperature at dilutions of 1/10 000 in Odyssey Blocking Buffer with 0.1% Tween-20 (Sigma-Aldrich) and 0.1% Tween. Imaging was conducted using the Li-Cor Odyssey Clx imaging system. Scans were performed at intensities that did not result in any saturated pixels.

2.4.13 Cycloheximide Protein Stability Assay

One million cells were plated for each time point and allowed to attach overnight. Cells were then cultured normally in atmospheric O_2 or in hypoxia (0.5% O_2) for 24h. Protein was extracted using M-PER (Thermo Fisher) as above. The protein was quantified by Pierce BCA Protein Assay Kit (Thermo Fisher) and measured on a FLUOstar Omega.
plate reader (BMG LABTECH; Offenburg, Germany). Twenty (20) µg of protein was analyzed by western blot using a FLAG antibody (Sigma-Aldrich).

2.4.14 Standard Curve Generation

After cDNA synthesis real time PCR was performed using Power SYBR Master Mix (Life Technologies). 1µL of the cDNA was loaded in triplicate for quantification of NODAL (1µL = 50ng of starting RNA).

NODAL forward primer: TACATCCAGAGTCTGCTG.
NODAL reverse primer: CCTTACTGGATTAGATGGTT.

Cloned NODAL PCR products were linearized, and diluted series were made (copy number/µL). These samples were used to construct a standard curve that was run with the experimental samples to estimate the number NODAL transcripts at each time point.

![Standard Curve of Linearized Nodal Vector](image)

**Figure 2.23 Standard Curve of Linearized Nodal Vector.**

Real-time PCR results for linearized Nodal vector one to 1 000 000 000 copies. Linear regression was used to determine the regression line. The equation of the line and $R^2$ value are provided.

2.4.15 In vivo Tumour Implantation

All experiments involving animals were approved by the Animal Use Subcommittee at the University of Alberta (AUP00001288 and AUP00001685).
Orthotopic Xenografts

A total of 500,000 SUM 149 cells in 100 µL RPMI:Matrigel (1:1) were injected into the right thoracic mammary fat pad of 7-8 week old female NSG mice. Mice were randomized, and treatments were administered when tumors reached a maximum diameter of 5 mm. At this point, mice were treated with DMSO vehicle control, INK (30 mg/kg by gavage), ISRIB (2.5 mg/kg IP) or paclitaxel (20 mg/kg IP or 15 mg/kg IV) for the times indicated. Tumour measurements were taken twice per week and a digital caliper was used to measure Length x Width x Depth of the tumour upon excision in order to calculate volume. Mice were sacrificed when tumors reached ~1 cm in diameter. Tumours were cut in half. One half was of the tumour was dissociated and the other was fixed with 4% formaldehyde, paraffin embedded, sectioned and stained with H&E or used for immunohistochemistry. Survival curves for overall survival were constructed using the Kaplan-Meier method and significance determined by log-rank test.

Patient Derived Xenografts

Two PDX models obtained through a collaboration with Oncotest (Charles River, Freiburg, Germany) were used: PDX 401 is a well differentiated basal-like TNBC and PDX 574 is a poorly differentiated basal-like TNBC. Viable pieces (~1mm in diameter) were placed, through a small incision, into the mammary fat pads of 7-8 week-old female NSG mice. At this point, mice were treated with DMSO vehicle control, INK (30 mg/kg by gavage), ISRIB (2.5 mg/kg IP or 10 mg/kg by gavage) or paclitaxel (20 mg/kg IP or 15 mg/kg IV) for the times indicated. Tumour measurements were taken twice per week and a digital caliper was used to measure length. Mice were sacrificed when tumours reached ~1 cm in diameter. One half of the tumour was dissociated and the other was fixed with 4% formaldehyde, paraffin embedded, sectioned and stained with H&E or used for immunohistochemistry. Survival curves for overall survival were constructed using the Kaplan-Meier method and significance determined by log-rank test.
2.4.16 Tumour Dissociation

Half of the 10mm tumours were fixed and paraffin imbedded for Immunohistochemistry. The other half of the tumour was dissociated using the MACS Miltienyi Biotec Human Tumour Dissociation Kit (MACS Miltienyi Biotec; Bergisch Gladbach, Germany) according to the manufacturer’s instructions prior to enumeration of live cells using trypan blue.

2.4.17 Flow Cytometry Identification of CSCs in Hypoxia and NODAL Overexpressing SUM149 Cells.

One million cells were stained in 100µL of Zombie Aqua (Fixable Viability Kit BioLegend; San Diego, California, USA) for twenty minutes at room temperature. Zombie Aqua was removed and 20µL of antibody dilution was added to each sample, which was then incubated on ice for 10-15 minutes.

Antibody Pairs:
- CD24 APC (1:20 dilution) (REA, Miltenyi MACS), CD44 Vioblu(e (1:5 dilution) (REA, Miltenyi MACS)
- FITC Mouse Anti-Human CD24 (1:5 dilution) (BD Biosciences; Franklin Lakes, New Jersey, United States), PE Mouse Anti-Human CD44 (1:5 dilution) (BD Biosciences)
- Human Carbonic Anhydrase IX/CA9 Fluorescein-conjugated Antibody (1:20 dilution) (R&D Systems, Minneapolis, Minnesota, United States), NODAL AF647 (1:20 dilution) (Novus Biologicals, Basel, Switzerland)

Cells were washed with 200µL FACs buffer (PBS with 10% FBS). Cells were pelleted at 1200 rpm for 3 minutes at room temperature and resuspended in 100µl of 2% PFA in FACs buffer. Samples were resuspended in another 300µL of FACS buffer for flow acquisition. Double discrimination and live cell gates were used to identify the cells of interest and quadrant gates were set according to the fluorescence minus one controls (FMO).
2.4.18 Polysome Profiling

Cells were grown to 60-80% confluence at which time time 0.1 mg/mL of cycloheximide was added to cells for 5 min at 37°C before harvesting. The cells were extracted in polysome lysis buffer (15 mM Tris·HCl (pH 7.4)/15 mM MgCl₂/0.3 M NaCl/1% Triton X-100/0.1 mg/mL cycloheximide/100 units/mL RNasein), and the volume of each lysate to be loaded onto gradients was determined by total RNA. Sucrose gradients (7- 47%) were centrifuged at 39,000 rpm with a SW41-Ti Rotor (Beckman Coulter, Fullerton, CA) for 90 min at 4°C. Gradients were continuously monitored at an absorbance of 254 nm and fractionated with a Brandel BR-188 Density Gradient Fractionation System. Each gradient was collected into nine equal fractions. The baseline absorbance of the sucrose gradient was calculated from the absorbance of a blank gradient using the Peakchart software and subtracted from the absorbance reading of each sample. RNA isolation was conducted by first digesting each fraction with proteinase K, and extracting total RNA by phenol-chloroform extraction and ethanol precipitation. Equal amounts of RNA were analyzed by real-time RT PCR.

2.4.19 Liquid Chromatography Tandem Mass Spectrometry

In-gel tryptic digestion of proteins separated on a 10% SDS-gel to produce tryptic peptides that were fractionated by a Waters nanoAcuity HPLC using an Acetonitrile linear gradient. Tandem mass spectrometry was performed on the system using waters trapping and analytical columns. Data from this experiment was analyzed using the FASTA UniProt database. For more detailed methods see Huges et al. [484].

2.4.20 Cloning

For cloning of the luciferase 5’ UTR reporters, we modified the PTK-ATF4-Luc plasmid from (Dey et al., 2010)[485] using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent; Santa Clara, California, USA) to introduce two BsmBI restriction sites flanking the ATF4 5’ UTR. The ATF4 5’ UTR was then replaced with a LacZ insert to complete the pGL3-TK-5UTR-BsmBI-Luciferase plasmid used for one-step cloning of various 5’ UTRs. This plasmid has been made available on Addgene (plasmid #114670). Except for “Snail 417”, 5’ UTRs of interest flanked by adapter sequences for cloning
were ordered as gBlocks from Integrated DNA Technologies (Coralville, Iowa, USA; sequences are listed in Supplemental Table 3) and cloned into pGL3-TK-5UTR-BsmBI-Luciferase using BsmBI (New England BioLabs; Whitby, Ontario, Canada). The “Snail 417” UTR insert was generated by PCR using the forward primer TATCGTCTCAACACCGAGC GACCCCTGCAATAAGCTTGCCGCTGAGCCGGTGGGC and the reverse primer ATACGTCTCTCTTTTATAGGTCAGGGACTGGGGGTGC. The “NODAL 298” uORF mutant was generated using the QuikChange Lightning Site-Directed Mutagenesis Kit with the forward primer CCTCCGGAGGGGGTTATATAATCTTAAAGCT TCCCGCAG and the reverse primer CTGGGGAAGCTTTAAGATTATATAACCCCTT CCCTGGAGG, to introduce a G-to-A mutation within an upstream start codon (ATG) at position -104 relative to the translational start.

2.4.21 Luciferase Assay

HEK293 cells were transfected using Polyethylenimine (PEI) (Sigma Aldrich; St. Louis, Missouri, USA). PEI and vector media were combined at a ratio of 5µL PEI and 0.5µg vector, 250µL of serum-free DMEM and incubated for 10min at room temperature. 250µL of the mixture was apportioned into each well of a 12-well plate containing 250µL DMEM. HEK293 cells were incubated overnight in the transfection mixture. The transfection media was removed and replaced with DMEM 10% serum and the cells were given 24h to recover. Cells were then treated with 0.1 µM of thapsigargin (TG) for 3h or 0.5% O₂ for 6 hours (hypoxia). Upon completion of treatment, cells were lysed and the luciferase activity measured with the Firefly Luciferase Assay System (Promega, Madison, Wisconsin, USA). Activity was read using the a FLUOstar Omega plate reader (BMG LABTECH).
References


Chapter 3

3 The Inhibition of mTOR and Cap-Dependence in Cancer Plasticity

3.1 Introduction

The mTOR pathway in cancer has been the subject of scientific and clinical inquiry for more than two decades now. Two different mTOR inhibitors have progressed through clinical trials though their efficacy is moderate, and their effects primarily cytostatic. These are the successes from more than 200 trials that had been registered with the National Health Institute [486]. There are a number of plausible reasons why these trials have not been successful including heterogenous expression of downstream effectors, microenvironmental factors, or pathway mutations [487-489]. Evidence from colon cancer shows that hypoxic cells are resistant to mTOR inhibition and that targeting of the hypoxia-regulated protein CA9 can diminish proliferation and spheroid formation from hypoxic cells [480]. There is reason to believe that concurrent inhibition of the mTOR and CA9 pathways would show complementary results, which is borne out in the Faes et al. study. CA9 likely inhibits mTOR function by altering the pH of the tumour microenvironment in response to hypoxia leading to mTOR resistance [480]. mTOR inhibitors may be more effective as combination therapies once their mechanisms have been better characterized.

mTOR is composed of 2 distinct complexes mTORC1 and mTORC2. The mTOR signalling pathway integrates cellular responses from growth factors, nutrients and energy availability to regulate cell survival, growth, proliferation, and motility [490]. The best studied downstream effectors of mTORC1 are Ribosomal Protein S6 Kinase β-1 (S6K1) and 4E-BP1 [466, 491]. When activated, mTORC1 phosphorylates S6K1, which in turn phosphorylates 40S ribosomal protein S6 (S6). This signalling acts to increase the translation of specific mRNAs like those possessing the 5`TOP sequence [492]. mTOR inhibition stops the phosphorylation of 4E-BP1 de-repressing it, allowing 4E-BP1 to bind eIF4E preventing the recognition of the 5`cap [493]. Hypophosphorylated 4E-BP1 binds to eIF4E to inhibit translation initiation and conserve resources, while allowing for the
translation of an alternate subset of mRNAs. Many important tumour promoting genes are regulated using this mechanism like HIF1α, VEGFA, BCL-2, c-MYC [245, 494-499].

The effects of stress-induced mTOR inhibition on plasticity and tumourigenicity are highly variable, suggesting that they may be particularly dependent on cancer type, microenvironment, or experimental model. In triple negative breast cancer, mTOR inhibition leads to the upregulation of mammospheres and CSCs [500]. As discussed earlier, conflicting data exists for the role of mTOR inhibition in cancer [501]. Additionally, it is difficult to discern from this work which mTOR regulated pathways are responsible for the observed phenotypes. More specific phenotypes found downstream of mTOR, like autophagy show more consistent results [502-504]. The effects of 4E-BP1 across multiple cancers is extensively reviewed by Musa et al. [505].

4E-BP1 is upregulated in nearly all tumour types [506]. It is also true that phosphorylated 4E-BP1 is upregulated in nearly every tumour type [505]. There is a notable absence in these data. The majority of the studies that measure the expression of phosphorylated 4E-BP1 did not measure the total level of 4E-BP1 [505]. These missing data are arguably the most essential missing experiments regarding 4E-BP1 expression in these population-level studies. Had total 4E-BP1 been included in these studies we would have been able to measure the amount and localization of active 4E-BP1. Without these data, we can only hypothesize as to why higher levels of an inactive protein would correlate positively with poor prognosis and tumour progression. The simplest explanation is that like healthy cells 4E-BP1 is produced at high levels and remains inactive to allow a rapid post-translational response to cytotoxic stress. More comprehensive data would allow us to make more precise inferences about the conditions under which 4E-BP1 activity promotes angiogenesis, chemoresistance, and tumour growth, and how those situations differ from when 4E-BP1 needs to be inactivated to allow for sustained proliferation and rapid tumour growth [245, 507-510]. In support of this idea three studies suggest that 4E-BP1, S6K1 and S6K2 are all overexpressed in breast cancer despite effecting opposite roles in mTOR signalling, and while all are independent prognostic factors in specific breast cancer populations, 4E-BP1 and S6K2 have stronger predictive value when used together [508-510]. These studies suggest a situational requirement for eIF4E dependent
and independent translation. Here I explore if stress signalling via the mTOR/4E-BP1 axis can increase plasticity and promote cancer progression.

3.2 Results

3.2.1 Inhibition of mTOR Increase Gene Expression Associated with Plasticity

Hypoxia and other physiological stresses alter the expression of thousands of proteins governing many facets of cellular physiology [511]. The inhibition of mTOR is not only an integral part of that response but a pathway that is of independent interest for its role in tumourgenesis, a subject about which many reviews have been authored [512]. To model the inactivation of mTOR that happens as a physiological response to stress, we treated SUM149 (Fig. 3.1 a) and T47D (Fig. 3.1 b) cells with 20nM of the mTOR inhibitor INK128 (MLN0128) for 3h and 6h. This dose of INK128 reduces mTOR’s kinase activity resulting in 4E-BP1 dephosphorylation promoting the active state. This activation can be observed by western blot as an increase in the low molecular weight hypophosphorylated 4E-BP1 band. Utilizing INK128, which should specifically inactivate mTOR, unlike hypoxia which effects numerous pathways, we still observe cross-talk between the mTOR/4E-BP1 and ISR pathways. INK128 increases the phosphorylation of eIF2α as measured by western blot analysis. The inactivation of ternary complex protein eIF2α that results from mTOR inhibition suggest that mTOR may act as a hub to coordinate these two pathways, adding to the list of pathways that converge on the ISR [513, 514].

Inhibition of the mTOR pathway increases the expression of genes associated with plasticity. In SUM149 cells the stem cell genes SOX2 and OCT4 are upregulated at both the level of transcription and translation (p<0.05, n=4). SNAIL and VIMENTIN, EMT genes, are also upregulated as seen by western blot and real-time RT-PCR (p<0.05, n=4). These changes in transcript abundance persist for one day after the INK128 treatment is removed. These same genes show a broader range of dynamics in T47Ds (Fig 3.2 b). Though NANOG, SOX2, OCT4, SNAIL, and TWIST increase at the protein level, SOX2
**Figure 3.1: mTOR Inhibition via INK128 Recapitulates the Activation of ISR Pathways.**

In (a) SUM149 and (b) T47D cells mTOR inhibitor INK128 increased the phosphorylation of eIF2α (Ser52), while simultaneously decreasing the phosphorylation of 4E-BP1 as measured by immunoblotting. eIF2α was measured by probing with an antibody to phosphorylate eIF2α and 4E-BP1 was measured as a change in size indicating a loss of phosphorylation. β-Actin and total eIF2α were used as a loading control. Cells were treated with 20nM INK128 for 3h and 6h.
Fold Change in Transcript Abundance (log\(_2\))

**SUM149**

- Control
- INK128 1 day
- INK128 1 day, 1 day recovery

**T47D**

- Control
- INK128 1 day
- INK128 1 day, 1 day recovery

**Markers**:
- NANOG
- OCT4
- SOX2
- SNAIL
- TWIST1
- VIM
- ZEB1

**Significance Levels**:
- * p < 0.05
- ** p < 0.01
- *** p < 0.001

**Western Blot Analysis**
- Sox2
- Oct4
- Snail
- Vimentin
- Actin
Figure 3.2: mTOR Inhibition Causes an Increase in EMT and Pluripotency-Associated Genes that Can Occur Independently from Transcriptional Increases.

(a) Real-time RT PCR analysis for transcripts of three pluripotency factors (NANOG, OCT4, SOX2) and four transcripts related to EMT (SNAIL, TWIST1, VIM, ZEB1) from SUM149 cells. Represented are the mean fold change above DMSO control ± SD. Two time-points were tested, 24h INK128 exposure (20nM), and 24h INK128 treatment followed by 24h in standard growth media. Four independent experiments were completed, and all mRNA increases were significant to a p-value < 0.005. Accompanying western blots of antibodies to SOX2, OCT4, SNAIL and VIM confirm the up-regulation of these proteins in response to 20nM INK128 after 24h and represent 3 independent experiments. β-Actin was used as a loading control.

(b) mTOR inhibition, real-time RT PCR and western blot analyses were repeated in T47D breast cancer cells, which showed an up-regulation in transcription of most factors (p<0.05). SOX2 was not upregulated in either condition, and TWIST1 returned to baseline after one day of recovery. Accompanying western blots probed with SOX2, OCT4, SNAIL, TWIST1 and VIM antibodies confirms the up-regulation these proteins in response to 20nM INK128 after 24h and represent 3 independent experiments. β-Actin was used as a loading control. The bars show the mean fold change ± SD. Asterisks indicate a significant difference by paired t-test (p<0.05, n=3).
does so independent of changes in transcription (p<0.05, n=4). The other transcripts *NANOG, OCT4, SNAIL,* and *TWIST* increase with 24h exposure to INK128. Given 24 hours to recover after INK128 treatment, by returning the cells to standard growth media, most of these mRNAs remain upregulated though *TWIST* expression returns to baseline. The inhibition of mTOR drives plastic gene expression in breast cancer cells.

Building on the concept that mTOR inhibition promotes plastic gene expression and that NODAL increases expression in response to hypoxia, we hypothesize that the inhibition of mTOR likewise upregulates NODAL even in the absence of the broader cellular stresses, and cell signalling that occur in hypoxia. As demonstrated by western blot analysis, NODAL expression is increased by INK128-mediated mTOR inhibition in a time-dependent manner measured at the 0h, 1h, 3h, 6h, 12h, and 24h time points (Fig 3.3). This increase was observed in two different cell lines: the T47Ds and the MCF7s. mTOR is a protein that regulates cellular physiology [515]. To explore the role that 4E-BP1 may play in regulating plasticity we generated four stably selected cell lines in both T47Ds and MCF7s. The four lines were a knockdown 4E-BP1 line made using a 4E-BP1 shRNA expressing vector (4EBP1sh), a 4E-BP1 overexpression cell line (+4EBP1), and two control lines containing either an empty vector (Empty) or scrambled shRNA control (SCR) (Fig 3.4). We confirmed the knockdown and the overexpression of 4E-BP1 by western blot. Notably, NODAL is upregulated in the cells overexpressing 4E-BP1.

### 3.2.2 Translation Inhibiting Signalling Along the mTOR/4E-BP1 Axis Increases Plasticity

Having demonstrated gene expression that is known to drive plasticity we measured if INK128 can induce the formation of CD44\textsuperscript{high}/CD24\textsuperscript{low} cancer stem cells. To assess if NODAL signalling could be part of the gene signature required for sphere formation we used the ALK 4/7 inhibitor SB431542 (10µM) to prevent signalling through the ALK4/7 and ACTR11B receptor complex. Of the three cell lines tested MCF7s demonstrated the most robust trends (Fig. 3.5 b). INK128 (20nM) caused a nearly 2-fold increase in CD44\textsuperscript{high}/CD24\textsuperscript{low} cells, and the addition of SB431542 caused a partial reversal of the
Figure 3.3: Inhibition of mTOR Increases Nodal Protein in Breast Cancer Cell Lines.

Western blot analysis probed for pro-Nodal ~39kDa using an anti-nodal antibody in two breast cancer cell lines (T47D and MCF7) tested after INK128 (20nM) treatment for 0h, 1h, 3h, 6h, 12h, and 24h. β-Actin was used as a loading control. Blots are representative of three separate experiments.
**Figure 3.4: Changes in the Expression of Translation Inhibiting 4E-BP1 Alter Nodal Expression.**

T47D and MCF7 cells were transfected with vectors containing either a 4E-BP1 shRNA, 4E-BP1, a scrambled shRNA or an empty vector and selected for stable expression. Western blotting was used to confirm the knockdown and the overexpression of 4E-BP1 with 4E-BP1 antibody in T47D and MCF7 cells. An increase in the expression of pro-Nodal, as measured by western blot, was observed in both 4E-BP1 overexpression cell lines.
Fold Change in CD44<sup>high</sup>/CD24<sup>low</sup> Cells

a) T47D

b) MCF7

c) SUM149

d) SUM149

Control | INK | INK SB

CD44 | CD24
Figure 3.5: Inhibition of mTOR Increase the $\text{CD44}^{\text{high}}/\text{CD24}^{\text{low}}$ Population of Breast Cancer Cells.

(a) T47D cells were treated with 20nM INK128 for 24h to assess changes in the number of $\text{CD44}^{\text{high}}/\text{CD24}^{\text{low}}$ cells. Conjugated CD44(PE) and CD24(FITC) antibodies were used to stain the cells before being counted by flow cytometry. This subpopulation increases relative to DMSO treated control (mean ± SD, t-test $p<0.05$, n=6). This effect was partially reversed with the addition of SB431542 (10 µM). (b) MCF7 cells were treated with 20nM INK128 for 24h to measure changes in $\text{CD44}^{\text{high}}/\text{CD24}^{\text{low}}$ cells. The cells displayed an increase in this subpopulation in response to INK128 (mean ± SD, $p < 0.05$, n=6) that was partially reversed by SB431542. (c) SUM149 cells were treated with 20nM INK128 for 24h, and the amount of $\text{CD44}^{\text{high}}/\text{CD24}^{\text{low}}$ cells were measured. mTOR inhibition increased the proportion of $\text{CD44}^{\text{high}}/\text{CD24}^{\text{low}}$ cells (mean ± SD, t-test $p<0.05$, n=6). Partial reversal of this effect was achieved with SB431542. (d) Representative scatter plots discriminating subpopulations as defined by cell surface markers CD44-PE and CD24-FITC for T47D cells. Isotype and fluorescence minus one controls were performed (not shown).
phenotype. The addition of SB431542 with INK128 significantly decreased CSC formation compared to the INK128 treated cells, but the number of CSCs remained elevated compared to controls (p<0.05, n=6). In both T47D cells (Fig. 3.5 a) and SUM149 cells (Fig. 3.5 c), INK128 increases the frequency of cancer stem cells. SB431542 causes a partial reversion of this phenotype (p<0.05, n=6). Figure 3.5 d shows representative scatter plots discriminating subpopulations as defined by cell surface markers CD44-PE and CD24-FITC for SUM149 cells. The inhibition of mTOR resulting from the administration of INK128 increases the abundance of CD44\textsuperscript{high}/CD24\textsuperscript{low} cells indicating an expansion of breast cancer stem cells. Part of this induction requires the signalling of ALK4/7 which transduces signals from members of the TGFβ superfamily including NODAL.

To address whether the changes in gene expression and CD44\textsuperscript{high}/CD24\textsuperscript{low} manifest as phenotypic changes, we used sphere formation to functionally measure putative CSCs. Increases in 4E-BP1 expression support plasticity and self-renewal \textit{in vitro}. In T47D cells the overexpression of 4E-BP1 increases first and second-generation sphere formation, demonstrating an increase in plasticity and self-renewal, respectively (Fig. 3.6 a). These data also suggest heritable alterations in the steady-state proportion of stem cells present in this population of T47Ds. Furthermore, the knockdown of 4E-BP1 reduces sphere formation in the second generation of spheres indicating an impairment of self-renewal. In the first-generation of spheres grown from the MCF7 cell lines, the overexpression of 4E-BP1 increases the sphere formation rate relative to the 4E-BP1 shRNA expressing line (Fig. 3.6 b). These differences increase in the second generation of spheres. The MCF7 cells overexpressing 4E-BP1 increase sphere formation relative to all groups in this experiment signifying an increase in self-renewal, plasticity and steady-state levels of CSCs. Figure 3.6 c shows images of spheres formed from the four T47D cell lines. In aggregate, these data clearly demonstrate that changes in the abundance of proteins that regulate translation can alter the dynamics of plasticity and self-renewal, which are substantive contributors to metastasis and tumour progression. As 4E-BP1 belongs to a family of eIF4E binding proteins capable of inhibiting translation it is possible that alterations in their expression levels could compensate for the changes in 4E-BP1.
Figure 3.6: Breast Cancer Cells Overexpressing 4E-BP1 Demonstrate Increased Plasticity and Self-Renewal.

Limiting dilution mammosphere assays were performed, for the T47D (a) and MCF7 (b) cell lines by seeding a 96 well ultra-low attachment plate with 0.5, 1, or 2 cells per well from a single cell suspension and allowing the mammospheres to grow for three weeks. Second generation mammospheres were generated by dissociating the primary mammospheres into a single cell solution and seeding 0.5, 1, or 2 cells per well. A significant increase above both controls was observed in first and second generation spheres produced from 4E-BP1 overexpressing T47Ds (+4EBP1) (p<0.05, n=3). Though MCF7 cells overexpressing 4E-BP1 generated more spheres than 4E-BP1 knock-down cells (4EBPsh), the increase was not significant when compared to both controls. 4E-BP1 overexpressing MCF7 cells significantly increased second generation sphere formation compared to all treatment groups (p<0.05, n=3). Different letters indicate test groups statistically different from one another by one-way ANOVA (p<0.05). Bars are the mean number of spheres formed ± SD. (c) Representative images of T47D mammospheres (250µM scale bars).
Figure 3.7: Breast Cancer Cells Exhibit a Nodal-Dependent Increase in Plasticity Under the Effects of mTOR Inhibition.

Sphere formation assay wherein, T47D (a), SUM149 (b) and MCF7 (c) were treated with DMSO, 20nM INK128 (INK), 100ng/mL rhNodal (Nodal), or 20nM INK128 and 10µM of SB431542 (INK SB). Limiting dilution mammosphere assays were performed by seeding 0.5, 1, or 2 cells per well from a single cell suspension into a 96 well ultra-low attachment plate. Sphere formation was measured at three weeks. INK128 increased sphere formation in all cell lines tested, as did Nodal. The effect of INK128 on sphere formation was returned to baseline by treatment with SB431542 (p<0.05, n=4). Horizontal lines indicate significant differences (one-way ANOVA). Bars are the mean number of spheres formed ± SD for four replicates. 250µm scale bars. (d) Representative images of T47D spheres from cells in each treatment group. Scale bar are 500µm.
Subsequently, we tested if INK128 (INK, 20nM) could increase sphere formation similar to that observed with the overexpression of 4E-BP1. INK128 was compared to the ability of rhNODAL (NODAL, 100ng/mL) to induce sphere formation. A fourth parameter was also tested in which INK128 (20nM) and SB431542 (10µM) (INK SB) were co-administered to test if signalling through ALK4/7 receptors mediated the changes induced by INK128. These parameters were tested in three cell lines: T47D (Fig 3.7 a), SUM149 (Fig 3.7 b), and MCF7 (Fig 3.7 c). In all three cell lines, INK128 and NODAL induce sphere formation to similar extents. It is also true that SB431542 is sufficient to block this induction in all our models. To that end, SB431542 in T47Ds reduces the level of sphere formation below that of the DMSO-treated control. Inhibition of mTOR in breast cancer cell lines can increase sphere formation to an extent similar to NODAL, which has been shown to promote plasticity in breast cancer. mTOR inhibition-induced plasticity requires signalling through ALK4/7 [516].

Concurrently, we tested the hypothesis that perturbations in mTOR/4E-BP1 signalling would alter anchorage-independent growth. To test this, we use a soft agar colony formation assay. In the T47D cells, we tested the four cell lines established in Figure 3.5: Empty, SCR, 4EBPsh, +4EBP1. The +4EBP1 lines increased anchorage-independent growth when compared to the knockdown line, 4EBPsh (Fig 3.8 a). When T47D cells were exposed to INK128 (20nM) for 24h, the colony formation rate doubled (Fig 3.8 b) (p<0.05, n=4). One hundred (100) ng/mL of rhNODAL also increases colony formation. Treatment with SB431542 in parallel with INK128 reverses the effect of INK128 on anchorage-independent growth, decreasing its levels below that of the controls. Similar experiments were carried out in the MCF7 cell lines. MCF7 cells overexpressing 4E-BP1 showed a robust increase in their ability to grow in the on agar (Fig 3.8 c) (p<0.05, n=4). Their colony formation is statistically different from both controls, Empty and SCR, and the knockdown line (4EBPsh). Knocking down 4E-BP1 had no effect on colony formation. Using INK128 to simulate stress-induced inactivation of mTOR, we observed that mTOR inhibition increases anchorage-independent growth (Fig. 3.8 d) (p<0.05, n=4). Likewise, 100ng/mL rhNODAL increases anchorage-independent growth in MCF7 cells. Administration of SB431542 suppresses the effects of mTOR inhibition on colony
Figure 3.8: mTOR Inhibition or Overexpression of 4E-BP1 Increases In vitro Anchorage-Independent Growth.

Soft agar colony formation assay. A suspension of single cells in 0.7% agar was plated on a layer of 1% agar and allowed to grow for ten days. (a) T47D 4E-BP1 overexpression cells showed an increase in colony formation compared to the 4E-BP1 shRNA expressing line (p<0.05, n=4). (b) 20nM INK128 and 100ng/mL rhNodal increased anchorage-independent growth. The effect of INK128 on colony formation was abrogated by treatment with SB431542 (p<0.05, n=4). (c) MCF7 4E-BP1 overexpression cells increased colony formation compared to both control lines, and the 4E-BP1 shRNA expressing line (p<0.05, n=4). (d) 20nM and 100ng/mL rhNodal increase anchorage-independent growth. The effect of INK128 on colony formation was abrogated by treatment with SB431542 (p<0.05, n=4). Horizontal lines indicate significant differences by one-way ANOVA. Bars are the mean fold change in colony number ± SD. (e) Representative images of fixed and stained soft agar colony forming assays. Triangle shows an individual colony.
formation. Like in the T47D cells this effect was potent enough to decrease soft agar colony formation below that of the vehicle group (DMSO) suggesting a baseline activation of this pathway in poorly aggressive breast cancer cell lines. Anchorage-independent growth is an important aspect of the breast cancer cell survival that can be upregulated by pluripotency factors like NODAL and pathways that regulate translation like the mTOR/4E-BP1 pathway.

3.2.3 In Vivo mTOR Inhibition Increases Plasticity in Part Through the Activation of the ISR.

There has been much enthusiasm for mTOR inhibitors as cancer therapies. Two inhibitors have successfully completed clinical trials and have been approved for use. These two treatments show modest but notable benefits for patients. As we demonstrated in Figure 3.1, mTOR inhibition also results in the activation of the ISR. Because mTOR responds to microenvironmental cues by regulating dozens of downstream pathways that ultimately control growth, proliferation, stress responses, and survival, mTOR inhibitors may cause heterogeneous and unreliable responses in tumours. If mTOR inhibitors can be combined with other treatments that cause a more uniform response, it may result in a more effective therapeutic approach. To test this possibility, we used a molecule called integrated stress response inhibitor (ISRIB) which binds eIF2B allowing for more efficient oligomerization of eIF2B subunits and enables ternary complex recycling even if eIF2α is phosphorylated. By limiting the in vivo endogenous activation of the ISR concomitant with limiting the mTOR induced activation of the ISR we may be able to gain the benefits of mTOR inhibition while sufficiently limiting the adaptive stress response to produce a more potent effect on tumour development. To test these effects in vivo, we injected 500,000 SUM149 cells into each mammary fat pad of NSG mice. The tumours were allowed to grow to 5mm before treatments were administered. Vehicle (DMSO), oral administration of INK128 (INK) (30mg/kg) or oral administration of INK in combination with intraperitoneal ISRIB (ISRIB+INK) (2.5mg/kg) were tested for their ability to reduce tumour growth. Drug treatments were administered every second day for two weeks, after that they were withdrawn, and tumours were allowed to grow to 10mm to
Figure 3.9: mTOR Inhibition In vivo Decreases Tumour Size.

NSG mice bearing bilateral SUM149 tumours were treated with DMSO as a control, INK128 (30mg/kg), ISRIB (2.5mg/kg) and INK128 (ISRIB+INK) every second day for two weeks starting when tumours reached 5mm. The rate of tumour growth was retarded in INK, ISRIB+INK treated mice as measured by tumour diameter of the longest axis. The data is represented by box and whisker plots (p<0.01, n=7). Individual tumours are marked by circles (o), individual means are marked by an x (x) and treatments statistically different from controls are indicated by horizontal lines.
Figure 3.10: Kaplan-Meier Plots Demonstrating Increased Survival of Tumour Bearing Mice Receiving INK128 Treatments.

NSG mice with SUM149 tumours treated with DMSO, INK128 (30mg/kg), and INK128 (ISRIB+INK) showed a significantly higher rate of survival than control DMSO treated mice (p<0.0001, n=6). Mice were sacrificed when tumours reached 10mm by caliper measurement along the longest axis. The probability of survival curves were calculated using the Kaplan-Meier product-limit method and compared via the log-rank test between treatment groups.
control for tumour size. By 12 days we observed growth retardation in both treatment groups (Fig. 3.9). The difference between treatment groups and the control group increased at 18 days (p<0.01, n=7). Size comparisons could not be made at later time points due to attrition in the treatment groups skewing the mean tumour size. There was no statistical size difference between the INK and ISRIB+INK treatment groups at the time points that could be tested. As INK128 limits tumour growth, one possible outcome of this experiment was that preventing the INK128 mediated ISR activation could have limited rather than enhanced the effects of mTOR inhibition. Here we are able to report that ISRIB does not prevent the growth inhibitory effects of INK128.

Though not producing a measurable effect on tumour size ISRIB did increase the survival of mice treated with INK128. Treatment every second day with INK128 also provided survival benefits determined by the Kaplan-Meier product-limit method and compared via the log-rank test (Fig. 3.10, p<0.0001, n=6). The median time from the beginning of treatment to sacrifice for the control (DMSO) group was 18 days, INK128 increased that time to 22 days, and the combination therapy mice survived 25 days. It is clear from these data that the combination therapy potentially holds therapeutic benefits by improving the survival of patients receiving anti-mTOR therapies.

Altered phenotypes manifested in the tumours may be underlying the observed changes in survival. Our in vitro experiments have shown that stress induces CSC generation and that translation inhibition via mTOR inactivation and eIF2α phosphorylation occurs as part of the survival response to multiple stresses. Many of the mRNAs that are responsible for the synthesis of proteins that support plasticity escape repression. We tested the tumours for changes in plasticity using a sphere formation assay. Cells were extracted from dissociated tumours, cleared of cell debris, and strained through a 40µm filter to attain single cell suspensions. Viable cells were counted and plated into 96 well ultra-low attachment dishes. Figure 3.11 shows the number of spheres formed per plate from tumours of mice receiving DMSO vehicle, INK128, or ISRIB+INK treatments. Despite decreasing tumour volume and improving survival, treatment with INK128 increased tumour sphere formation suggesting countervailing pathway activation that may limit the effectiveness of these strategies. Co-treatment of INK128 and ISRIB
Figure 3.11: Addressing Treatment-based Increases in Cancer-stem-cells (CSC) with ISRIB.

Tumoursphere formation assay showing that inhibition of mTOR via INK128 induces CSC formation that is reversible with ISRIB. After growing to 10mm, in NSG mice, the SUM149 tumours were removed dissociated, filtered, and the viable cells counted. One hundred cells were seeded per well of a 96 well non-adherent plate. The treatment of SUM149 tumours by INK128 (30mg/kg) resulted in an increase in CSCs as measured by sphere formation (p=0.0011). ISRIB (2.5mg/kg) counteracted this increase, reducing the number of spheres below control levels (p=0.0001, Control n=12, INK n=13, ISRIB+INK n=13). Horizontal lines indicate significant differences (one-way ANOVA). Bars are the mean number of tumourspheres ± SD. (b) Representative images of spheres from each treatment. Scale bars are 500µm.
Figure 3.12: In Vivo Expression of ATF4 Resulting From the Administration of INK128 can be Controlled by ISRIB.

(a) The number of ATF4 expressing SUM149 cells (brown stained nuclei) in immunohistochemically stained tumour sections from NGS mice treated with DMSO, INK128 (30mg/kg), or ISRIB (2.5mg/kg) and INK (ISRIB+INK). INK increases the number of ATF4 high cells, but when combined with ISRIB this number returned to control levels. The asterisk (*) indicates a significant difference from all other groups (ANOVA). Bars are the mean number of ATF4 positive cells per field of view ± SD. Three random fields of view were counted for each tumour. (b) Representative images are shown for each treatment (p<0.05, n=3).
reduces sphere formation below that of the control. In this model of breast cancer, SUM149, and at a dose of 2.5mg/kg, ISRIB was capable of reducing the induced sphere formation caused by INK128. The effect was potent enough to decrease the sphere formation to 50% of the endogenous sphere forming capacity of the control tumours. Figure 3.11 b contains representative images of the spheres formed from each tumour. To verify the effects of ISRIB on the tumours and to assess the activation of the ISR we tested the expression of ATF4 in fixed and paraffin embedded tumour sections by counting the number of cells that expressed high levels of ATF4 in three fields of view (Fig. 3.12). INK128 increased the frequency of high ATF4 expressing cells, as evidenced by dark brown staining, by two-fold indicative of the activation of the ISR. When ISRIB was combined with INK128 the previously observed increase in cells expressing high levels of ATF4 was wholly abrogated. These data provide in vivo evidence that inhibition of mTOR results in the activation of the ISR and that the inhibitor of eIF2α phosphorylation prevents this signalling which in turn alters the adaptive protein response including decreasing ATF4 expression.

Plasticity has been identified as a crucial characteristic of metastasizing cells including circulating tumour cells. We assessed the potential role of mTOR inhibition in promoting extravasation and seeding in the lungs of NSG mice by pretreating cells with either DMSO or INK128 and injecting them into the tail vein of the mice. Three weeks later the lungs of the mice were removed, fixed, sectioned and stained with human HLA to identify human breast cancer cells (brown staining) (Fig. 3.13 c). Tumours found in the lungs of ten mice were counted by two scientists and reported as the number of tumours per section (Fig. 3.13 a). SUM149 cells that were pretreated with INK128 were twice as likely to colonize the lung when compared to DMSO treated control cells (p<0.05, n=10). INK128 tumours not only formed with a higher frequency but also grew to a greater size than those derived from control cells. Typical tumours for each treatment can be seen in Figure 3.13 c. Inhibition of mTOR increases the ability of SUM149 cells to survive in the circulatory system, extravasate into the lung, and develop into tumours at a secondary site. These experiments illustrate that the cells have become proficient at these steps of the metastatic cascade.
Figure 3.13: INK128 Alters the Dynamics of Metastasis to the Lungs.

Lung metastasis assays were performed to address the potential of INK128 to increase metastasis. (a) 500,000 SUM149 cells or SUM149 cells treated with 20nM INK128 were intravenously injected into the tail vein of NSG mice. Eight weeks post-injection the lungs were sectioned and stained for human HLA to identify the presence of any human tumour cells. INK128 increased seeding in the lungs (Student’s t-test, p<0.05, n=10). The asterisk (*) indicate significant differences calculated using Student’s t-test. Individual mice are marked by circles (○). (b) Percentage of mice with detectable micrometastases in the non-treatment and treatment group. (c) 20x bright field microscope images of micrometastases formed in each treatment group.
**Figure 3.14: Quantification of Cellular Fraction of the Necrotic Regions in Tumours Treated With INK, ISIRB and ISRIB+INK.**

ISRIB decreased the area of cells within necrotic regions of the tumour compared to both DMSO control and ISRIB+INK treatments in SUM149 tumours from NSG mice. Each dot represents one unique tumour that was analyzed for percent hypoxic area which was measured using ImageJ and is presented as percentage of the total area. To determine the hypoxic area the total tumor section area was selected and subsequently measured by thresholding against background pixel intensity. Second round of thresholding against lighter necrotic tumor regions was used to measure total area of necrosis. Cellular regions were determined by dense dark areas within necrotic regions, where cells have intact nuclei. All areas were measured in pixels and all threshold selections were manually examined during analysis to ensure correct assignment of necrotic or cellular necrotic regions. Hematoxylin and eosin stain (H&E) stained tumour section from 4 treatment groups DMSO control, INK128 (30mg/kg), ISIRB (2.5mg/kg), or ISRIB (2.5mg/kg) and INK128 (ISRIB+INK). Statistically different relationships determined Student’s t-test, p<0.05)
If ISRIB is effective at decreasing stress responses, this may impact the tumour’s ability to withstand low oxygen, potentially altering the amount of stress response within a tumour. Tumour sections from DMSO control (n=9), INK128 (30mg/kg, n=12), ISRIB (2.5mg/kg, n=12), or ISRIB (2.5mg/kg) and INK128 (30mg/kg, ISRIB+INK, n=10) were hematoxylin and eosin (H&E) stained. This staining allows for three values to be determined via image analysis, which allows us to calculate the cellular area within the necrotic region as a fraction of the total tumour area. The three regions are the total area of the section, the necrotic area, and the nuclei positive fraction of the necrotic area, the cellular area within the necrotic region. The results are presented as the percent of the total tumour composed of cells in necrotic regions. Tumours from mice receiving DMSO had the greatest proportion of cells in necrotic areas (Fig. 3.14). INK128 decreased the mean necrotic cellular area, but the tumours showed dramatically different responses revealing itself in the high variability of this group. This group contained both the most and the least responsive tumours. ISRIB and ISRIB+INK mice had more uniform responses both demonstrating a decrease in necrotic cellular area relative to the total volume of the tumour. ISRIB showed the greatest decrease in the mean necrotic cellular area of the four conditions. Representative tumour sections are shown for each of the tested conditions. These tumours show structural differences from one another in their cellularity in necrotic areas. It has been demonstrated in many ways that hypoxia is associate with disease progression, so cells better capable of surviving in harsh microenvironment may show similar aggressive characteristics [517]. These data suggest a potential biophysical mechanism though which ISR inactivation could limit tumourigenesis.

*In vivo* analysis of tumour tissue using IHC was employed to test if 4E-BP1 activation was occurring in regions of hypoxia. To do this, serial sections of SUM149 tumours were stained with an anti-4E-BP1 antibody, anti-phospho-4E-BP1 antibody, and CA9 antibody. Activation of 4E-BP1 occurs where total 4E-BP1 is present and phosphorylated 4E-BP1 is not detectable. These areas of active 4E-BP1 correlate with the expression of CA9 providing evidence that translation inhibition is occurring as a means of adapting to hypoxia in tumours derived from SUM149 cells (Fig. 3.15). Extensive 4E-BP1 activation is observable in both groups, which suggests that the majority of 4E-BP1 activation is the
Figure 3.15: Regions of Hypoxia Contain Active 4E-BP1 in SUM149 Tumours.

Immunohistochemically stained serial sections of SUM149 tumours treated with vehicle (DMSO) or INK128 (30mg/kg) probed for total 4E-BP1, phosphorylated 4E-BP1, and CA9 (brown). CA9 expression, an indicator of hypoxia, was found highest around the margins of 4E-BP1-P and areas where phosphorylated 4E-BP1 was absent. Images reflect three replicates, insets show similarly sized hypoxic structures within the tumour. Scale bar = 1000µm, scale bar inset = 250 µm.
result of endogenous tumour stress. The high variance in the abundance of hypophosphorylated 4E-BP1 makes changes in 4E-BP1 resulting from INK128 treatment challenging to determine. Power analysis (α=0.5, power=0.9) estimates 300 tumours per condition to attain significance (sections from three tumours can be found in Supplemental Figure 3). The relative signal to noise makes this particular experiment impractical for the value it provides. The insets show similarly sized hypoxic structures in which hypophosphorylation occurs. We have established the activation of 4E-BP1 in the hypoxic regions of SUM149 tumours confirming activation of a central stress response that we and others have linked to various tumourigenic phenotypes.

Cancer cell lines have been the cornerstone of cancer research for decades. Though cell lines have been instrumental in the preclinical development of cancer treatments and are often the best available model, they have rather poor performance as preclinical models [518]. PDXs offer a new model that eliminates some of the issues that derive from selecting cells that grow in monolayers. PDXs have been shown to recapitulate the cellular and histological structure of the original tumour better, which are essential aspects of stress and hypoxia [519]. PDXs better maintain the genomic and genetic profiles of the tumours from which they derive [520]. Most importantly for our study, there is a high degree of correlation between PDXs’ responses to therapy and the donors’ clinical responses [521, 522]. Evaluation of the two models makes it clear that PDXs offer a more robust portrait of tumour heterogeneity than breast cancer cell lines. To further capture tumour heterogeneity we use two different PDX models, PDX401 and PDX574.

Mice bearing bilateral PDX401 tumours were treated with vehicle (DMSO) or 30mg/kg INK128 (INK) orally every second day for two weeks. After the final treatment, tumours were allowed to recover and grow to 10mm in diameter to control for tumour size in the follow-up experiments. By day 18 there was a small but significant increase in tumour size in the mice treated with INK128. Unlike the SUM149 cell line (Fig 3.16), growth retardation does not occur in the PDX401, worse still, it is enhanced by INK128 treatment.
Figure 3.16: INK128 Treatment Increased the Size of PDX401 Tumours.

NSG mice bearing bilateral PDX401 tumours were treated with DMSO or INK128 (30mg/kg) every second day. Treatment was initiated upon tumours reaching 5mm in diameter and administered for two weeks. The rate of tumour growth was enhanced in INK128 treated mice as measured as tumour diameter along the longest dimension. Data presented as box and whisker plots (Student’s t-test p<0.01, n=8). Individual tumours are marked by circles (○) and treatments statistically different from controls are indicated by horizontal lines.
Figure 3.17: Measuring Treatment-based Increases in Cancer-stem-cells (CSC) Induced by INK128.

INK increases tumoursphere formation. The 10mm PDX401 tumours were dissociated, filtered, and the cells were counted. In each well of a 96 well non-adherent plate, cells were seeded at a concentration of 100 cells per well. The oral administration of INK128 (30mg/kg) to mice with PDX401 tumours increases CSCs as measured by sphere formation (p=0.023, DMSO n=6, INK n=7). Bars are the mean number of tumourspheres ± SD, and an asterisk indicates a statistical difference (*). Significances was determined by Student’s t-test. Images are representative of mammospheres formed. Scale bars are 500µm.
Figure 3.18: CA9 and Nodal Staining in PDX401 and PDX574.

Immunohistochemical staining for CA9 and NODAL (brown) expression in the well-differentiated PDX401 lines and in poorly-differentiated PDX574. PDX 574 expresses higher levels of both CA9 and NODAL.
Previously we demonstrated that even when INK128 is effective at decreasing tumour size other protumorigenic phenotypes like sphere formation are enhanced, therefore we measured sphere formation in the PDX401 tumours from control and INK128 treated mice. Cells dissociated from 10mm tumours were plated at a concentration of 100 cells per well of a 96 well low attachment plate. The tumours that were treated with INK128 showed an increased ability to form spheres in non-adherent conditions (Fig 3.17) (p=0.023, DMSO n=6, INK n=7). The photomicrographs are representative images of the spheres formed in each condition. Here we demonstrate that INK128 fails as monotherapy for the treatment of PDX401 tumours, worsening two clinical criteria. It is notable though that INK128 did not decrease the survival of the mice according to Kaplan-Meier product-limit method and compared via the log-rank test (n=4).

We have classified the PDX401 tumours as low hypoxia tumours according to their CA9 expression (Fig 3.18). In the INK128 and control (DMSO) tumours CA9 expression is low, but there is still a considerable amount of un-phosphorylated 4E-BP1, as determined by areas containing high 4E-BP1 and low 4E-BP1-P (Fig 3.19). This dephosphorylation likely arises from an uninvestigated stress like nutrient deprivation. Many CA9 expressing regions absent of 4E-BP1 phosphorylation are present in the tumour such as those shown in the insets of Figure 3.18. There did not seem to be an overall change in 4E-BP1 phosphorylation between the two treatments (Supplemental Figure 3). Immunohistochemical characterization of tumours in this manner provided valuable insights into the stress responses present in the tumour and therein the variance in their utilization in each tumour.

We also tested the effects of INK128 on the growth of PDX547. This PDX showed the highest level of CA9 expression of the PDXs that we evaluated (Fig. 3.18). These tumours grew more rapidly than those derived from PDX401. Four treatments were administered to the mice: DMSO, ISRIB (2.5mg/kg), INK128 (30mg/kg), and the combination of ISRIB and INK128 (ISRIB+INK) (Fig 3.20). Tumour measurements began at a size at which they could be reliably attained, a diameter greater than 4mm. ISRIB does not have a statistically significant effect on tumour size, but the small sample size of the experiment prevents clearly ruling out a potential benefit. INK128 and
Figure 3.19: Hypoxic Regions of PDX401 Tumours Contain Active 4E-BP.

Serial sections of immunohistologically stained PDX401 tumours, previously characterized as CA9 low, treated with vehicle (DMSO) or INK128 (30mg/kg) stained with total 4E-BP1, Phosphorylated 4E-BP1 (4E-BP1-P), or CA9 antibodies (brown staining). Tumour CA9 was mostly absent, but areas of unphosphorylated 4E-BP1 were common. Images reflect 3 replicates insets show similarly sized hypoxic structures within the tumour. Scale bar = 1000µm, scale bar inset = 500 µm.
Figure 3.20: Tumour Growth and Survival of Mice Bearing PDX547 Tumours Receiving INK128 Treatment.

Groups treated with INK128 and INK128 with ISRIB showered retarded growth and greater survival than control mice (DMSO). DMSO, INK128 (30mg/kg), ISRIB (2.5mg/kg) and ISRIB with INK128 (ISRIB+INK) were administered every second day for two weeks to mice possessing PDX574 mammary fat pad tumours starting when tumours reached 5mm. The rate of tumour growth was retarded in INK128, and ISRIB+INK treated mice as measured by tumour diameter of the longest axis (p<0.001, n=6) (a). Individual tumours are marked by circles (○), and treatments statistically different from controls using one-way ANOVA are indicated by horizontal lines. (b) Mice bearing PDX574 tumours treated with INK128, or ISRIB and INK128 (ISRIB+INK) showed significantly higher survival as compared to DMSO or ISRIB treated mice (p<0.0001, n=6). Mice were sacrificed when tumours reached 10mm. The probability of survival curves were calculated using the Kaplan-Meier product-limit
INK128 in combination with ISRIB both effectively decreased tumour size (Fig 3.20 a). ISRIB neither enhanced nor hindered the effects of mTOR inhibition on tumour size. PDX574 tumours and the SUM149 (Fig. 3.9) cell line derived tumours both responded to INK128 whereas PDX401 (Fig. 3.16) did not. We gain a valuable insight from these data, which would have been missed had we not tested a second PDX model. Resistance to INK128 treatment is not a result of the more representational mutational profile, tissue structure or any other aspect native to PDXs. If that were true, PDX574 would have had a similar increase in growth. It must then result from some patient tumour specific response. INK128 and INK128 in combination with ISRIB are effective at controlling the growth rate of some tumours, with the important caveat that we would not expect all patients to respond indicating the need for an accompanying diagnostic to improve patient selection.

Mice in these experiments were treated every second day for two weeks or until the tumour reached 10mm in diameter along its longest axis. Mice in the ISRIB and DMSO groups received fewer treatments due to the rapid growth of the tumours; this is unlikely to have had an effect on the experiment as the tumours had, by day ten, achieve statistical differences in size. Two treatments resulted in significant improvements in survival: INK128 and INK128 with ISRIB (ISRIB+INK) (Fig. 3.20 b). The population size of this experiment was insufficient to rule out that the changes between DMSO and ISRIB, or INK and ISRIB+INK were due to chance.

Upon staining serial sections of the tumours with 4E-BP1, 4E-BP1-P and CA9 antibodies it becomes clear that these tumours possess a structural and hypoxic landscape distinct from that of the SUM149 or the PDX401 tumours. The tumours extensively express CA9 (Fig. 3.20). The CA9 clearly borders the areas of the tumour that lack 4E-BP1-P, thus delineating the margins of areas undergoing mTOR inhibition. Many areas that lack phosphorylated 4E-BP1 have intermediate CA9 expression and are surrounded by high levels of CA9 membrane staining. Hypoxia is a much more prominent feature of these tumours compared to PDX401. In these tumours, the correlation between the hypoxia marker CA9 and the lack of 4E-BP1 phosphorylation suggest that hypoxia-induced cellular stress is responsible for much of the mTOR inhibition observed.
Figure 3.21: Hypoxic Regions of PDX574 Tumour Contain Active 4E-BP.

Immunohistochemical staining of serially sectioned PDX574 tumours from NSG mice, characterized as CA9 high. Tumours were treated with vehicle (DMSO) or INK128 (30mg/kg) stained with total 4E-BP1, Phosphorylated 4E-BP1, or CA9 (brown) antibodies. Tumour CA9 was heterogeneously expressed in the tumours, areas positive for 4E-BP1 but lacking p-4E-BP1 showed moderate CA9 expression and areas with extensive p-4E-BP1 showed a notable absence of CA9. Images reflect 3 replicates, insets show similarly sized hypoxic structures within the tumour. Scale bar = 1000µm, scale bar inset = 500µm.
Figure 3.22: Analysis of RNA-seq from 1100 Breast Cancer Patients (TCGA).

(a) Expression of the 4E-BP1 transcript increases in tumours above levels found in healthy adjacent breast tissue using RNA-Seq by Expectation Maximization (RSEM) to estimate transcript abundance (Student’s t-test, p<0.001). (b) A Kaplan-Meier plot demonstrating the correlation between 4E-BP1 expression and survival (Wilcoxon, p=0.0187). Low levels of 4E-BP1 predict survival within the first 2000 days (5.4 years). Low EIF4EBP1 (n=150), High EIF4EBP1 (n=845).
To help predict if mTOR inhibition and translation inhibition are features of breast cancer at a population level, we analyzed the RNA-seq data from 1100 breast cancer patients and compared the expression of 4E-BP1 (EIF4EBP1) to that in normal adjacent breast tissue. 4E-BP1 is significantly upregulated in primary lesions (Fig 3.22 a). To determine if 4E-BP1 expression correlated with survival, patients were dichotomized according to their 4E-BP1 expression by receiver operating characteristics (ROC) curves to determine the optimal cutoff for the endpoint of overall survival censorship. When these two groups are compared using the Kaplan-Meier product-limit method low 4E-BP1 expression is predictive of overall survival for the first 2000 days (5.4 years) post-diagnosis. These data combined with the mixed clinical, in vivo, and in vitro evidence for mTOR inhibition indicate that mTOR inhibition may be more successful as a combination therapy than a monotherapy. Alternatively, mTOR inhibition could be used as a targeted therapy against vulnerable tumours. Because mTOR inhibition and active 4E-BP1 are intrinsic characteristics of tumour cell signalling, gaining a clearer understanding of how tumours respond to further translation inhibition by mTOR inhibitors is necessary to predict which patients that will receive the greatest therapeutic benefits.

3.3 Discussion

As we have previously shown, stress can narrow translation to a fraction of the levels found in non-stressed cells (Fig 2.9). Within the remaining translation-competent mRNAs are those that are required for plasticity and EMT. This ultimately leads to increases in in vitro assays that test for surrogates of tumourigenic phenotypes like sphere formation. There are many stresses that can affect translation: hypoxia is one that is also a recurrent feature of solid tumours [523]. Protection from hypoxia requires such a rapid and dramatic cellular response that many of the necessary proteins essential to adaptation are synthesized at high levels under normal conditions including HIF1α, 4E-BP1 and PERK but inactivated through proteasomal degradation, phosphorylation and inhibitor binding, respectively [203, 524-526]. Stress conditions release repressive signalling, which facilitates an immediate response without the need to transcribe and translate new proteins. The ability to immediately limit translation is an integral aspect of surviving in hypoxia and conserving energy, of which mTOR and 4E-BP1 are crucial actors by way
of inhibiting cap binding [527, 528]. The other major pathway responsible for translation inhibition is PERK/eIF2α, the activation of which prevents ternary complex formation.

We first demonstrate that there is cross-talk between these two pathways (Fig 3.1). Inhibition of mTOR using INK128 increases the phosphorylation of eIF2α. As previously detailed mTOR and 4E-BP1’s roles in cancer are not clearly defined. The effect of mTOR inhibitors may be different depending on pathway activation, mutational profile, or microenvironmental factors the balance of which may determine which of mTOR’s many divergent roles are most prominently activated [529-532]. By limiting the activation of pro-survival pathways, like the ISR, which activate as a result of mTOR inhibition, we may be able to tailor the outcomes of mTOR inhibition towards its anti-tumour effects and prevent some of the pro-tumourigenic adaptations that can occur in a subset of tumour cells.

To explore this concept, we tested to see if mTOR inhibition can produce a pluripotent gene signature. This response also suggests that mTOR inhibition may be one mechanism by which hypoxia up-regulates stemness. We demonstrate that INK128 increases gene expression of EMT and stem cell genes (Fig 3.2). We also show that INK128 upregulates NODAL in a similar fashion to that seen in hypoxia in breast cancer cell lines (Fig 3.3). This change in NODAL may also drive plastic phenotypes. We showed that exposure to INK128 could up-regulate CD44<sup>high</sup>/CD24<sup>low</sup> breast CSCs in an ALK4/7 dependent manner (Fig. 3.5). This exposure to INK128 increases sphere formation and anchorage-independent growth more than NODAL did alone (Fig. 3.7 and 3.8 b and d). Overall, inhibition of ALK4/7 was able to entirely reverse the effect on both phenotypes with some cell line variability. In T47D cells sphere formation was suppressed below that of the control, suggesting that ALK4/7 activity is part of the innate signalling that promotes sphere formation in these lines. In both cell lines, anchorage-independent growth was reduced to half of the control signifying a prominent role of ALK4/7 in the regulation of this phenotype. It is clear from these data that intermittent mTOR inhibition can increase plasticity.
$\log_2(\text{TPM}+1) \text{ mTOR}$
Log$_2$(TPM+1) EIF4E
$\log_2(\text{TPM} + 1) \ 4E-BP2$
Pan-cancer analysis of TCGA using Gepai. Data is presented as gene expression (Log2(TPM+1)) of tumour (T) in red and normal tissue (N) in green. Statistically significant changes greater than 0.5-fold with a p-value less than 0.01 are indicated by the colour of the cancer abbreviation (red – upregulated in tumour tissue, green – downregulated in tumour tissue, and black – the gene remaining unchanged). 4E-BP1 (a) is the most consistently upregulated of the investigated genes, followed by EIF4E (c). mTOR (b) and 4E-BP2 (d) show no consistent pattern of expression between cancer types. (e) Key containing TCGA cancer type abbreviations.

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<th>Abbreviation</th>
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<td>Kidney Chromophobe</td>
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<td>brca</td>
<td>Breast invasive carcinoma</td>
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<td>Lymphoid Neoplasm Diffuse Large B-cell Lymphoma</td>
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<td>Kidney renal clear cell carcinoma</td>
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Figure 3.23: Expression of 4E-BP1, EIF4E, mTOR, and 4E-BP2 Across Cancer Types In TCGA.
Another mechanism by which the mTOR/4E-BP1 pathway is altered is by the overexpression of 4E-BP1. Using the data available in the TCGA we have shown that increased 4E-BP1 expression is a feature of many cancers, being more consistently upregulated than EIF4E, mTOR, or 4E-BP2 (Fig 3.23). By stably transfecting cells with 4E-BP1 overexpression vectors we demonstrated increased 4E-BP1 expression, like that seen in many cancers, can cause the upregulation of NODAL (Fig 3.4). Overexpression of 4E-BP1 increased plasticity and self-renewal in the MCF7 and T47D cells as measured by first and second-generation sphere formation (Fig. 3.6). The same overexpression cell lines showed an enhanced ability to grow in soft agar colony formation assays (Fig. 3.8 a and c).

One of the more consistent effects of mTOR inhibition on cancer is the inhibition of growth [533]. We observe that oral dosing of INK128 inhibits tumour growth. Despite eIF2α phosphorylation being induced by INK128 the co-administration of ISRIB to prevent that phosphorylation did not adversely affect INK128’s growth suppression (Fig. 3.9). INK128 had no discernable effect on survival but INK, when used in combination with ISRIB, increased survival compared to controls from 18 days to an average of 24 days (Fig. 3.10). We investigated if the administration of INK128 or ISRIB was altering other phenotypes of the tumour. First, when we explored sphere formation, we discovered that, like in vitro, INK128 increases plasticity resulting in a 60% greater rate of sphere formation (Fig. 3.11). More importantly, we found that the use of ISRIB in combination with INK128 substantially impaired the plasticity of cells from SUM149 tumours. ATF4, a central ISR survival protein which is regulated at the level of translation by eIF2α phosphorylation, is upregulated by INK128 and this increased expression is prevented by co-treatment with ISRIB (Fig. 3.12). The increase in plasticity and ATF4 expression show two different mechanisms by which the effectiveness of mTOR inhibition as a cancer therapy could be diminished. Though it will undoubtedly take a significant amount of research to understand the breadth of mTOR signalling, impeding the activation of the ISR with ISRIB is one means of preventing the co-activation of a pro-tumourigenic pathway that regulates multiple neoplastic phenotypes.
We employed a tail vein assay to assess if mTOR inhibition may affect other tumour phenotypes such as extravasation and seeding at secondary sites (Fig. 3.13). Monolayer cultured cells treated with DMSO or INK128 were injected into the tail vein of mice and allowed to migrate to and seed in the lung. INK128 treated cells more effectively colonized the lungs compared to DMSO treated control cells. Not only was the average number of micrometastases increased but the number of mice in which a tumour formed more than doubled. Tail vein experiments with these types of inhibitors can be done one of two ways: pretreating the cells, or treating the mice after injections. We utilized the former strategy, which is designed to test if exposure to stress signalling can prime metastatic potential. The latter experimental design would test if systemic administration of INK128 could be protective against metastasis. Though exciting, the latter question remains unanswered. Our data do suggest that intermediate exposure to mTOR inhibition from INK128, or potentially from hypoxia or nutrient deprivation, can prime cells allowing them to metastasize more readily.

Hypoxia employs eIF2α phosphorylation and mTOR inhibition to facilitate a survival program [534]. Administration of ISRIB compounds disrupts the tumour’s ability to sustain regions of hypoxia. ISRIB and ISRIB in combination with INK128 reduce the total area of hypoxia in SUM149 tumours (Fig. 3.14). Hypoxia is a negative prognostic factor, which was reduced by the two most effective treatments in these tumours, though it is not possible from these data to determine if the relationship is functionally relevant, it is a promising relationship. With this data, we also confirmed, in vivo, that areas of the tumour surrounded by the hypoxia marker CA9 displayed decreased 4E-BP1 phosphorylation indicating inactive mTOR and active 4E-BP1 (Fig. 3.15). Less commonly we observed areas encircle by CA9 expression that also possessed 4E-BP1 phosphorylation and areas without boarding CA9 expression that lacked 4E-BP1 phosphorylation. This heterogeneity of activation would likely limit the effect of mTOR inhibitors as they would presumably be less effective in areas in which mTOR is not signalling.

We tested two different PDX models to see if they recapitulated what was observed in the SUM149 cell line. These PDXs differed in both their NODAL expression and their CA9
expression (Fig. 3.18). PDX401 had low levels of CA9 and NODAL expression, and PDX574 had higher levels of these proteins (Fig. 3.18). Upon further histological examination of PDX401 we determined that despite expressing lower levels of CA9, the amount of active 4E-BP1 did not dramatically differ from that of PDX574 or SUM149 tumours (Fig. 3.19). This discrepancy suggests that either CA9’s expression is not an adequate histological marker of hypoxia in some tumours, or that the PDX401 tumours have extensive non-hypoxia driven mTOR inhibition. In both PDX models, there were CA9 positive areas in which active 4E-BP1 — positive for 4E-BP1 total/negative for phosphorylated 4E-BP1 — were identifiable. These two PDXs also differed in their response to INK128. PDX401 was made worse by INK128, where tumour size, and sphere formation both increased (Fig. 3.16, Fig. 3.17). These changes were moderate resulting in no change in survival (data not shown), but this type of change would normally be undetectable in a large-scale clinical trial where two different treatments cannot be tested in the same patient and thus a small number of patients who respond differently than the majority would be conflated with those who are resistant or have more rapidly progressing diseases. The results in PDX574 mirrored what we observed in SUM149 tumours. INK128 and the combination of INK128 and ISRIB both decreased tumour size but were not significantly different from one another (Fig. 3.20 a). Likewise, both INK128 and the combination therapy improved survival, but the sample size was insufficiently powered to determine if these two groups were statistically different from one another (Fig. 3.20 b). These tumours clearly express much higher levels of CA9, which border areas of low 4E-BP1 phosphorylation indicating that in these tumours hypoxia may be a much more prominent driver of mTOR inhibition than the previous PDX model (Fig. 3.21). Using three distinct models gives us a much more relevant view of the spectrum of possible responses to INK128 as a therapy then we would have had, had we used only one, especially if that one had been a cell line. However, having tested only three models there is insufficient evidence to determine why one PDX responds to mTOR inhibition-based therapy and the other does not. From these data, it appears that the amount of hypoxia may be one factor that determines the effectiveness of mTOR inhibition as a therapy, but that hypothesis requires systematic evaluation to be verified.
Figure 3.24: Analysis of RNA-seq from 1085 Breast Cancer Patients
Demonstrating Dysregulation of Members of the mTOR Signalling Pathway (TCGA) and Their Prognostic Value.

Analysis of data from 1085 breast cancer patient samples and 291 normal samples from the TCGA using Gepai. (a) Data is presented as gene expression, Log₂(TPM +1), tumour tissue is presented in red and non-tumour tissue gray. EIF4E is upregulated in tumour tissues, mTOR is unaltered and 4E-BP2 is downregulated (Student’s t-test, p<0.05). (b) Kaplan-Meier plots demonstrating the relationships between EIF4E, mTOR, or 4E-BP2 and five-year survival for patients dichotomized by median expression into two equal groups (n=201). No gene demonstrated a significant correlation between expression and survival.
Figure 3.25: 4E-BP1 is Dysregulated Relative to the Other mRNAs in the mTOR Pathway.

Correlational analysis of gene expression from 1085 breast cancer patient samples and 291 normal samples from the TCGA using Gepai to assess dysregulation of the mTOR pathway. Data is presented as scatterplots of gene expression, Log2(TPM + 1). R-value and p-value as calculated by linear regression are included with each graph. The most dramatic changes from normal to tumour occur in correlations containing 4E-BP1.
Figure 3.26: Analysis of TCGA Breast Cancer Data for the Frequency and Type of Mutations in Genes Related to ISR and mTOR/4E-BP1 Signalling.

Mutational analysis of 'All Complete Tumors (n=994)', paired tumour and normal samples from the Breast Invasive Carcinoma dataset (TCGA, PanCancer Atlas) using cBioPortal. Both the frequency and type of mutation are presented as a portion of the total cohort (gray). EIF4EBP1 is mutated at more than twice the rate of any other gene in the analysis. For all genes, except for mTOR, the prevailing types of mutations are amplifications and mRNA upregulating mutations.
Our analysis of 4E-BP1 expression from the over one thousand patients in the TCGA database suggests that 4E-BP1 is overexpressed in most cancers and that it is a better predictor of early survival than other components of the signalling pathway (Fig. 3.22, Fig. 3.24) [535, 536]. 4E-BP1’s correlations with mTOR and eIF4E that exist in healthy tissues are diminished in cancer (Fig. 3.25). Furthermore, 4E-BP1 is the most commonly mutated of the interrogated translation regulating genes with the majority of the mutations being mRNA upregulating and amplifications (Fig. 3.26) [535, 536]. These data indicate that 4E-BP1’s disruption in cancer may represent an unmet treatment need.

mTOR signalling has a central role in many phenotypes often acting as a gatekeeper to promote survival or induce apoptosis [537-539]. Like many other aspects of mTOR/4E-BP1 biology the effects reported in the literature on cancer phenotypes are mixed [245, 500, 501, 505, 506]. Our data is no exception to this. We observe both pro- and anti-tumourigenic changes that result from the inhibition of mTOR. A model that requires balanced expression may best explain these complex results. The complexity of the data makes it clear that hypotheses such as those that state that mTOR signalling or loss of 4E-BP1 expression are pro-tumourigenic, or conversely those that state that active 4E-BP1 contributes to tumourigenesis are too simple. More in-depth exploration of the components of this pathway suggests that ratios of components are essential to responses [540]. Whether it is the ratio of 4E-BP1 to phospho-4E-BP1, or the ratio of 4E-BP1 to eIF4E, the relative abundance of actors in these pathways may be crucial in determining which effects arise more prominently from mTOR inhibition [505, 540]. Though not contradicted, the ratio explanation is confounded by 4E-BP1, and eIF4E expression changes in response to hypoxia, which act to alter these ratios, meaning that what is true for the normoxic cells may not be true for the hypoxic cells [541]. In vivo experiments in which there is a spectrum of microenvironments, including varying degrees of hypoxia, would potentially respond less uniformly resulting in dramatically different outcomes than the ones observed in the more homogenous microenvironments found in in vitro experiments.

Another complicating factor is that many models use the expression of constitutively active 4E-BP1, gene deletions or other long-acting cellular alterations as part of their
experimental designs, which may have unintended consequences on a system that requires balanced expression or time-dependent alterations in gene expression [542, 543]. These types of more static experimental models, when not explicitly recapitulating observed changes in patient tumours, cannot adequately replicate the dynamics of the mTOR and hypoxia response systems [234]. The translational hypoxic response is sequential and phased, the responses of which are dependent on the duration of insult [234]. It is reasonable to expect a different response from models that depend on more permanent alterations in expression given that both hypoxic signalling and mTOR inhibition produce different responses contingent on the duration of the stimuli [544-546]. mTOR inhibition induces 4E-BP1 hypophosphorylation with acute exposure that subsequently becomes hyperphosphorylation with continued exposure to rapamycin [544]. Models of persistent and acute hypoxia, likewise, show time-dependent expression of different hypoxia-regulated genes [547]. Many experimental designs are unlikely to capture these dynamics, nevertheless the data may provide vital mechanistic insights, these caveats must be considered.

A paper by Ding et al. provides the best evidence yet for the divergent roles of 4E-BP1 in cancer. The paper has two key findings: first that 4E-BP1 is required for hypoxia tolerance — proliferation and apoptosis resistance — and that the loss of 4E-BP1 increases the rate at which neoplastic progression occurs. From these data, and the fact that heightened 4E-BP1 expression is a feature of nearly every tumour type in the TCGA database, we hypothesize that expression 4E-BP1 must be maintained, even if inactive, at a level that, when needed, is partially protective against hypoxia, but low enough that clonal selection and tumour progression still occur. Because 4E-BP1 is inactive when it is not being utilized for survival, high levels of its expression do not have to interfere with cell growth. A hypothetical model in which mTOR activity or eIF4E expression is high enough that 4E-BP1 does not limit cell growth, but 4E-BP1 is present at sufficiently high levels to overcome hypoxic stress, should the need arise, can reconcile many of the seemingly contradictory observations about mTOR and 4E-BP1 in cancer. The totality of the data suggests that high levels of translation support rapid growth and thus higher levels of 4E-BP1 are required to inactivate translation and elicit metabolic adaptations to overcome stresses like hypoxia [548].
Ultimately, we require a theoretical model that allows for the pro-growth contributions of mTOR signalling, the pro-survival role of active 4E-BP1, which also reconciles hypoxia as a driver of tumour progression, and finally fits with the expression patterns and survival presented (Fig 3.21). Once we have this model, we can begin to design falsifiable hypotheses and refine them, which may elucidate how to enhance mTOR and 4E-BP1 derived therapies. The combined effect of mTOR inhibition, while abrogating the potential pro-tumourigenic adaptations that result from the activation of the ISR, is an early but significant step in understanding the role of mTOR inhibition in breast cancer and designing better therapeutic options to address mTOR signalling.

3.4 Methods

3.4.1 Cell Culture and Treatments

T47D cells, obtained from ATCC (Manassas, Virginia, USA), were maintained in RPMI-1640 Medium (Life Technologies; Carlsbad, California, USA) with 10% fetal bovine serum (FBS) (Life Technologies) at 37°C with 5% CO₂. Cells were passaged using 0.25% (w/v) trypsin (Life Technologies) as per ATCC recommendations. MCF7 cells, obtained from ATCC, were maintained in RPMI-1640 Medium with 10% FBS at 37°C with 5% CO₂. Cells were passaged using 0.25% (w/v) trypsin as per ATCC recommendations. SUM149 cells, purchased from BioreclamationIVT, were grown in Ham's F-12 medium with 5% heat-inactivated FBS, 10mM HEPES, 1µg/mL hydrocortisone, and 5µg/mL insulin. All cells were grown in a humidified environment at 37°C with 5% CO₂.

H9 hESCs from WiCell (Madison, Wisconsin, USA) grown on irradiated CF-1 Mouse Embryonic Fibroblasts (GlobalStem; Gaithersburg, Maryland, USA) in knockout DMEM/F12 (Life Technologies; Carlsbad, California, USA), 20% knockout serum replacement (Life Technologies), 1X non-essential amino acids (Life Technologies), 2mM glutamine (Life Technologies), 0.1mM 2-mercaptoethanol (BME; Thermo Fisher Scientific; Waltham, Massachusetts, USA), and 4ng/mL of basic fibroblast growth factor (FGF) (Life Technologies). For experiments cells were passaged into feeder-free
conditions. Feeder free conditions consisted of Geltrex matrix (Life Technologies) as a growth substrate and mTeSR1 media (Stem Cell Technologies; Vancouver, British Columbia, Canada). We employed recombinant human NODAL (R&D Systems, Minneapolis, Minnesota, United States) and SB431542 (10µM) (Sigma-Aldrich) to activated and inhibit NODAL signalling as well as INK128 (MLN0128, 20nM) (Sellekchem Houston, TX USA).

3.4.2 Hypoxia Treatments
Hypoxia was administered at the noted concentrations using Xvivo system (BioSpherix; Parish, New York, USA). The Xvivo’s continuous monitoring was used to ensure consistent and accurate oxygen levels were maintained. Though different oxygen concentrations were used for different experiments, the temperature and CO₂ were consistent at 37°C with 5% respectively. Upon the completion of the hypoxia treatment, cells were removed and processed for downstream application.

3.4.3 Sphere Formation
Sphere formation media was composed of DMEM/F12 + GlutaMax (Life Technologies), 1x B27 (Life Technologies), 20 ng/mL epidermal growth factor (EGF) (Life Technologies), and 10 ng/mL FGF (Life Technologies). After treatment, cells were harvested using 0.25% (w/v) trypsin (Life Technologies), the trypsin was neutralized, and the cells resuspended in fresh media. These cells were filtered through a 40µm pore filter (Thermo Fisher) to obtain a single cell solution. Cells were counted using trypan blue (Thermo Fisher) and diluted in the sphere formation media to the appropriate concentration for plating. Seed 200µL of the diluted cells into each well of a 96 well Ultra-Low Attachment Surface plates (Corning, NY, USA). Spheres were given between 10 and 21 days to grow.

3.4.4 Imaging Spheres
Images of spheres were captured using the EVOS FL Cell Imaging System (Thermo Fisher) at 4X magnification. Minimal changes were made to contrast and brightness to improve the visibility of the spheres.
3.4.5 RNA Extraction

The PerfectPure RNA Cultured Cell Kit (5-Prime; Hilden, Germany) was used to extract total RNA from cultured cells following the manufacture’s protocol using the following optional methods. Lysis buffer was added directly to the plate (option c for cell lysis manufacturer’s instructions). Optional DNase treatment was performed, and RNA was eluted in 50\textmu{}L. Three (3) \textmu{}L of purified RNA was used for quantification using the Epoch plate reader (Biotek; Winooski, Vermont, USA).

3.4.6 Reverse Transcriptase PCR and Complementary DNA Synthesis.

cDNA was made from purified total RNA using high capacity cDNA reverse transcription kit (Applied Biosystems; Foster City, California, USA) as per manufacturer’s protocol. The included random hexamers were used to prime reverse transcription and 1\mu{}g of RNA was used for each. ‘No Template,’ reactions without RNA and ‘No RT,’ a reaction without reverse transcriptase enzyme were included as controls.

3.4.7 Real Time PCR Analysis of Gene Expression

Real time PCR analysis was performed on 1\textmu{}L of cDNA using TaqMan Gene Expression Master Mix according to the manufacturer’s procedures. FAM labelled TaqMan® gene expression human primer/probe sets (Thermo Scientific). mRNA expression was compared to untreated control using the ΔCT method. Data was collected on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad; Hercules, California, USA) using standard real time PCR settings.

1) Activation 95\textdegree{}C 10 min
2) Melting 95\textdegree{}C 15 sec
3) Annealing/ extension 55\textdegree{}C 1 min. Return to step 2 for 40 total cycles

Melt curve analysis was performed to ensure the production of a single amplicon.
3.4.8 Plasmid Preparation

One Shot™ TOP10 Chemically Competent E. coli were transformed according to the manufacturer’s instructions and grown for 16h in liquid culture with the appropriate antibiotic for selection (50μg/mL kanamycin; (Life Technologies), 100μg/mL ampicillin; (Life Technologies)). Cells were pelleted at 3000g and purified using Geneaid Midi Plasmid Kit (Endotoxin Free) (Geneaid Biotech; Taipei City, Taiwan) as per manufacturer’s instructions. For all primers see Supplemental Table 1.

3.4.9 Lipofectamine 2000 Transfection

To increase 4E-BP1 levels we used an expression vector (pCMV6-Entry EIF4EBP1 True ORF Gold Vector; OriGene). To knock down 4E-BP1 we used pGFP-V-RS EIF4EBP1 Human shRNA (OriGene) versus scramble and control vector. Cell lines were transfected with plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacture’s instructions. Stable cell lines were selected with 500µg/mL until all sham transfected cell had died. Cells were maintained in 250µg/mL of G418).

3.4.10 Western Blots

Cells were lysed on-plate using Mammalian Protein Extraction Reagent (M-PER; Thermo Scientific), with Halt Protease Inhibitor Cocktail (Thermo Scientific), and Phosphatase Inhibitor (Thermo Scientific). Protein was quantified according to manufacturer’s instructions utilizing Pierce BCA Protein Assay Kit (Thermo Fisher) and measured on a FLUOstar Omega plate reader (BMG LABTECH; Offenburg, Germany). 4x Laemmli buffer (Bio-Rad; Hercules, California, USA) with 5% BME (Sigma-Aldrich; St. Louis, Missouri, USA) containing 20µg of protein was boiled for 10 minutes and loaded to be analyzed. Samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE), and then transferred onto Immobilon-FL membranes (Millipore). Membranes were blocked with 5% milk in PBS 0.1% Tween (Sigma-Aldrich) for 1h at room temperature, incubated with primary antibody overnight at 4°C (Supplemental Table 2). After the membranes were washed in PBS 0.1% Tween (Sigma-Aldrich) horseradish peroxidase (HRP)-conjugated secondary antibodies (Bio-Rad) were applied. The membranes were incubated for 1h at room temperature, washed to remove
excess secondary antibody, and Clarity Western ECL Substrate (Bio-Rad) was used to detect signal. ChemiDoc™ XRS+ System (Bio-Rad) or film were used to image the western blots. Densitometry was performed using ChemiDoc™ XRS+ System (Bio-Rad).

Florescence western blot detection: Using the Trans Blot Turbo (settings of 25 V and 1.3 A for 15 minutes; Bio-rad) proteins were transferred to a low-auto-fluorescence PVDF membrane (Bio-rad), blocked for one hour at room temperature with Odyssey Blocking Buffer (Li-Cor; Lincoln, Nebraska, USA), then incubated with primary antibody overnight at 4°C in Odyssey Blocking Buffer with 0.1% Tween-20 (Sigma-Aldrich). Membranes were then probed with corresponding Li-Cor anti-mouse or anti-rabbit fluorescent secondary antibodies for one hour at room temperature at dilutions of 1/10 000 in Odyssey Blocking Buffer with 0.1% Tween-20 (Sigma-Aldrich) and 0.1% Tween. Imaging was conducted using the Li-Cor Odyssey Clx imaging system. Scans were performed at intensities that did not result in any saturated pixels at each time point.

3.4.11 In vivo Tumour Implantation

All experiments involving animals were approved by the Animal Use Subcommittee at the University of Alberta (AUP00001288 and AUP00001685).

Orthotopic Xenografts

A total of 500,000 SUM 149 cells in 100 µL RPMI:Matrigel (1:1) were injected into the right thoracic mammary fat pad of 7-8 week old female NSG mice. Mice were randomized, and treatments were administered when tumors reached a maximum diameter of 5 mm. At this point, mice were treated with DMSO vehicle control, INK (30 mg/kg by gavage), or ISRIB (2.5 mg/kg IP) for the times indicated. Tumour measurements were taken twice per week and a digital caliper was used to measure Length x Width x Depth of the tumour upon excision in order to calculate volume. Mice were sacrificed when tumors reached ~1 cm in diameter. Tumours were cut in half. One half was of the tumour was dissociated and the other was fixed with 4% formaldehyde, paraffin embedded, sectioned and stained with H&E or used for immunohistochemistry.
Survival curves for overall survival were constructed using the Kaplan-Meier method and significance determined by log-rank test.

**Patient Derived Xenografts**

Two PDX models obtained through a collaboration with Oncotest (Charles River, Freiburg, Germany) were used: PDX 401 is a well differentiated basal-like TNBC and PDX 574 is a poorly differentiated basal-like TNBC. Viable pieces (~1mm in diameter) were placed, through a small incision, into the mammary fat pads of 7-8 week-old female NSG mice. At this point, mice were treated with DMSO vehicle control, INK (30 mg/kg by gavage), or ISRIB (2.5 mg/kg IP or 10 mg/kg by gavage) for the times indicated. Tumour measurements were taken twice per week and a digital caliper was used to measure length. Mice were sacrificed when tumours reached ~1 cm in diameter. One half of the tumour was dissociated and the other was fixed with 4% formaldehyde, paraffin embedded, sectioned and stained with H&E or used for immunohistochemistry. Survival curves for overall survival were constructed using the Kaplan-Meier method and significance determined by log-rank test.

**Tail-Vein Lung Metastasis Assay**

SUM 149 cells were pre-incubated as described, then trypsinized and counted. 500,000 cells in 700 uL Ca2+-free HBSS were injected into the tail vein of female NOD-scid IL2Rgamma null (NSG) mice. Mice were sacrificed at 8 weeks (to tumour formation). Lungs were formalin-fixed and paraffin-embedded, and immunohistochemical staining on this tissue was conducted using a human-specific HLA antibody (Supplementary Table 1) as per the manufacturer’s instructions. For each mouse organ, 3-6 sections were acquired from evenly spaced areas throughout the tissue, and the average number of metastases per mouse organ was calculated.

3.4.12 Tumour Dissociation

Half of the 10mm tumours were fixed and paraffin imbedded for Immunohistochemistry. The other half of the tumour was dissociated using the MACS Miltienyi Biotec Human Tumour Dissociation Kit (MACS Miltienyi Biotec; Bergisch Gladbach, Germany)
according to the manufacturer’s instructions prior to enumeration of live cells using trypan blue.

3.4.13 Flow Cytometry Identification of CSCs in Hypoxia and NODAL Overexpressing SUM149 Cells.

One million cells were stained in 100µL of Zombie Aqua (Fixable Viability Kit BioLegend; San Diego, California, USA) for twenty minutes at room temperature. Zombie Aqua was removed and 20µL of antibody dilution was added to each sample, which was then incubated on ice for 10-15 minutes.

Antibody Pairs:
- CD24 APC (1:20 dilution) (REA, Miltenyi MACS), CD44 Vioblue (1:5 dilution) (REA, Miltenyi MACS)
- FITC Mouse Anti-Human CD24 (1:5 dilution) (BD Biosciences; Franklin Lakes, New Jersey, United States), PE Mouse Anti-Human CD44 (1:5 dilution) (BD Biosciences)

Cells were washed with 200µL FACs buffer (PBS with 10% FBS). Cells were pelleted at 1200 rpm for 3 minutes at room temperature and resuspended in 100µl of 2% PFA in FACs buffer. Samples were resuspended in another 300µL of FACS buffer for flow acquisition. Double discrimination and live cell gates were used to identify the cells of interest and quadrant gates were set according to the fluorescence minus one controls (FMO).

3.4.14 Immunohistochemical Staining

Staining was performed using the Envison+System HRP anti-mouse IgG (Dako; Santa Clara, California, USA) Formalin-fixed, paraffin-embedded tissue underwent deparaffinization in xylenes, hydration through an ethanol series, antigen retrieval with citrate buffer (Dako), and peroxidase and serum-free protein blocking (Dako). NODAL, HLA, CA9, p4E-BP1, 4E-BP1 or ATF4 specific antibodies (Supplementary Table 2) were applied. Slides were rinsed in TBS-T and treated with Envison+ HRP anti-mouse IgG (Dako). Color was produced with DAB (brown) substrate (Dako) and counterstained with Mayer’s haematoxylin (Vector Laboratories, Burlingame, California, USA). Samples were dehydrated in reagent grade alcohol and cover slipped with permanent
mounting medium. Negative control reactions were conducted with mouse IgG, isotype controls used at the same concentration as the primary antibodies.

3.4.15 Analysis of Patient Data

Level 3 TCGA RNAseqV2 BRCA gene expression data and clinical information was obtained from the TCGA Data Portal in April 2015. RNA-sequencing RSEM values were used in downstream analyses. For TCGA RNA-seq samples, relative abundance (transcripts per million, TPM) was calculated by multiplying the scaled estimate data by 106 and used in downstream analysis. We conducted all analyses and visualizations in the RStudio programming environment (v0.98.501). R/Bioconductor packages ggplot2, survminer, pROC, and survival were used where appropriate. 4E-BP1 expression was dichotomized with receiver operating characteristics (ROC) curves to determine the optimal cutoff for the endpoint of overall survival censorship. Student’s t-test was used to evaluate expression differences between cohorts. The associations between 4E-BP1, and overall survival were tested in univariate Cox regression models with 4E-BP1 considered as continuous variables (as log [RSEM expression values+1] or binarized by ROC curves). Survival curves for overall survival were constructed using the Kaplan-Meier method and significance determined by Wilcoxon test.
References


Chapter 4

4 ISR Facilitates Chemotherapy Induced Plasticity

4.1 Introduction

Exposure to hypoxia, be it acute or chronic, has a profound effect on tumour growth, treatment resistance, and metastasis. These effects are widely observed in multiple cancers and at multiple stages of the disease, suggesting that it is not just the intrinsic features of low oxygen tension like adduct formation, or drug diffusion distance that are responsible for the effects of hypoxia, but that hypoxia fundamentally alters tumours propelling them toward more tumourigenic phenotypes. PERK and eIF2α are principal regulators of this process and are responsible for reducing energy consumption by restricting both protein folding and protein synthesis while upregulating the expression of cytoprotective gene sets [549-551]. eIF2α is the convergence point for multiple pathways which cumulatively are called the integrated stress response (ISR) [513]. The most studied of these kinase pathways in hypoxia is part of the unfolded protein response.

PERK is an ER membrane protein that under normal conditions is bound to its inhibitor BiP [552, 553]. Upon accumulation of unfolded protein in the ER BiP dissociates from PERK resulting in its activation [552]. PERK then phosphorylates eIF2α causing an increase in affinity for eIF2B while eIF2 is bound to GDP [358, 359]. These eIFs bound in this way cannot form the ternary complex thus inhibiting translation [358, 359]. Finally, genes like ATF4 are upregulated at the level of translation to protect the cell [554].

PERK and eIF2α play an essential role in the establishment of solid tumours by increasing survival under hypoxic conditions. This effect is dependent on the phosphorylation of eIF2α. Survival in hypoxia allows for the establishment of larger tumours, which was partially mediated by ATF4 expression [555]. eIF2α inhibition improved the chemoresistance of hypoxic cells via protection from ROS and induction of autophagy independent of HIF1α [556].
Several laboratories have reported the activation of the PERK/eIF2α pathway to be a characteristic of CSCs. Chronic exposure to hypoxia induces a stem cell state concomitant with sustained UPR activation and quiescence in metastatic breast cancer [77]. In a comparison of MCF7 CSCs to the bulk population, eIF2 signalling was the most overrepresented pathway suggesting a preferential need for these genes [557]. Breast CSCs derived from bone metastases show gene expression patterns indicating the importance of EMT and active UPR signalling in metastasis. In this study, there is also a strong correlation between the UPR and EMT gene signature supporting the idea that UPR activation may support EMT based plasticity [410]. Additional support of PERK/eIF2α regulating plasticity comes from work from gastric cancer in which severe hypoxia induces EMT in an eIF2α dependent manner [558]. CSCs also require eIF2α phosphorylation to successfully transition from mono-layers to 3D culture in sphere formation assays. This eIF2α phosphorylation also protected against stress-induced apoptosis. Inhibition of the UPR sensitized sphere-forming cells to chemotherapy [559]. PERK/eIF2α were protective against anoikis — another form of cell death which occurs with the loss of cell adhesion of ordinarily adhesive cells. During the process of matrix detachment, tumour cells use the activation of the UPR and ATF4 expression to increase migration, invasion, reduce anoikis and increase metastasis [403]. Though the evidence for the contribution of PERK/eIF2α signalling and its role in tumour progression is still in the early stages, these data and those collected in ‘normal’ models like ESCs demonstrate that PERK/eIF2α can drive the acquisition and maintenance of plasticity. In this chapter, I will explore the hypothesis that eIF2α phosphorylation drives plasticity and chemoresistance and that this process is targetable in vivo. Preventing adaptive responses to stress by blunting the activation of the UPR/ISR my be an effective strategy for improving therapeutic efficacy.
Results

4.1.1 ER Stress Induces a CSC Phenotype in an eIF2α Dependent Manner.

Stress is an integral aspect of tumour development driving clonal selection. The broader the range of the microenvironmental pressures the more aggressively selection occurs [215, 560]. Both hypoxia and chemotherapy drive this errant evolutionary process [215, 561]. A central strategy to survival and maintenance of energy homeostasis in response to hypoxic stress is to regulate mRNA translation, a response which was characterized in chapter one. Many stress responses have proven to be general rather than specific. A phenomenon like HIF1α response to chemotherapy or heat shock protein expression in hypoxia provide examples of the shared role for stress pathways in response to multiple stresses [272, 562]. In chapter one we demonstrated that the chemotherapeutic agent paclitaxel activated the pathways that are responsible for the downregulation of translation in hypoxia — eIF2α and mTOR (Fig. 2.12). Our subsequent investigation of translational efficiency and the role of mTOR inhibition established a link between translation and plasticity. To test what role, if any, the UPR has in regulating plasticity we treated MCF7 and T47D cells with azetidine-2-carboxylate (5mM, AZE), a known inducer of UPR, for times ranging from 1h to 24h and measured the amount of NODAL protein by western blot (Fig 4.1). Induction of the UPR increases the amount of NODAL, which we have previously shown stimulates CSC phenotypes. This response was observable in both cell lines. In order to focus on the PERK arm of the UPR, we used Salubrinal, another inducer of UPR, which prevents the dephosphorylation of eIF2α by inhibiting eIF2α phosphatase enzymes [563]. Salubrinal, like INK128, increased eIF2α phosphorylation and NODAL expression as measured by western blot analysis with an antibody to eIF2α’s phosphorylated at S51 (Fig. 4.2). ISRIB enables ternary complex formation even in the presence of phosphorylated eIF2α by activating eIF2B [564, 565]. This activation, in turn, replenishes the ternary complex pool, preventing translation inhibition [566]. ISRIB decreases NODAL expression (Fig. 4.2). Salubrinal induces a broader induction of pluripotent gene expression measured as increases in NANOG, SOX2, SNAIL and SLUG by western blotting at 0, 3 and 6h (Fig 4.3). Multiple experimental methods of inducing ISR and eIF2α result in an increase in the expression
Figure 4.1: ER Stress Increases Nodal Protein in Breast Cancer Cell Lines.

Western blot analysis confirmed elevated pro-Nodal ~39kDa expression induced by azetidine-2-carboxylate (5mM, AZE), an activator of the unfolded protein response in two breast cancer cell lines (T47D and MCF7) measured at time 0h, 1h, 3h, 6h, 12h, and 24h. Western blots were probed with an anti-Nodal antibody. β-Actin was used as a loading control. Blots are representative of three separate experiments.
Figure 4.2: Inhibition of mTOR by INK128 Causes the Phosphorylation of eIF2α.

Western blot analysis confirmed the increase in the phosphorylation of eIF2α in response to 20nM INK128. Western blots were probed with eIF2α-p and Nodal antibodies to determine changes in expression. INK128 induced phosphorylation of eIF2α (eIF2α-p) was similar to that of 10 µM of Salubrinal after 6h. INK128 and Salubrinal upregulated pro-Nodal (Nodal). The images represent three independent experiments. β-Actin and eIF2α were used as a loading control.
Figure 4.3: Inhibition of Ternary Complex Formation by Salubrinal Causes an Increase in Pluripotency-Associated and EMT Genes in T47D cells.

(a) Western blots confirmed the up-regulation of NANOG, SOX2, SNAIL, and SLUG in response to 10 µM Salubrinal at 3h and 6h. Western blots were probed with β-ACTIN, NANOG, SOX2, SNAIL, and SLUG antibodies. The images represent three independent experiments. β-ACTIN was used as a loading control.

(b) Real-time RT PCR analysis for transcripts *NANOG* and *SNAIL* from T47D cells. Represented are the mean fold change relative to DMSO control ± SD. Two time-points were tested 3h and 6h (n=4).
of genes regulating plasticity.

To better understand the role of eIF2α in the induction of plasticity we used three cell lines derived from MDA-MB-231 cells. The two control lines were transfected with empty vector (EV) and wildtype eIF2α (WT) and a third that was transfected with a vector containing eIF2α with a Serine 51 to Alanine 51 mutation (KI) to prevent eIF2α phosphorylation. Cells were exposed to TG at a concentration of 0.1μM to induce UPR/ISR stress (Fig 4.4) [567]. In the two control lines, eIF2α phosphorylation and ATF4 expression increased. When eIF2α was rendered incapable of being inactivated via phosphorylation, the increase in ATF4 expression was attenuated (Fig 4.4). ATF4 was chosen to verify the effect of these cell lines because it is known to be translationally regulated, which we verified in chapter one. When these same cell lines were exposed to hypoxia and subjected to sphere formation assays, the control lines (EV and WT) both increased the number of spheres they formed (Fig. 4.5 a). Conversely, without the ability to regulate eIF2α phosphorylation, the KI line lost the ability to increase sphere formation in response to hypoxia (Fig. 4.5 a) (p<0.05, n=3). Representative images of the spheres are found in Figure 4.5 b. Plasticity is the result of broad changes in gene expression that occur at the level of transcription and translation. We tested for changes in gene expression that could contribute to hypoxia-induced plasticity using real time RT-PCR to measure the abundance of SNAIL, TWIST, VIM, and ZEB in untreated cells and those cells exposed to hypoxia (0.5% O₂) for 24h (Fig. 4.5 c). We found that TWIST, VIM and ZEB were upregulated in response to hypoxia in the wild-type line, but when the phosphorylation site of eIF2α is mutated, VIM and ZEB upregulation is lost, and TWIST is downregulated in response to hypoxia. In a model of breast cancer where eIF2α phosphorylation is strongly abrogated, hypoxia-induced plasticity as measured by sphere formation and gene expression is ablated providing evidence that the regulation of translation via eIF2α upon exposure to stress is a plausible driver of the acquisition of a cancer stem cell phenotype.
Figure 4.4: Verification of an MDA-MB-231 Cell Line Deficient in eIF2α Phosphorylation by Western Blot.

MDA-MB-231 cells containing phosphorylation-defective eIF2αS51 (Mut) have impaired eIF2α phosphorylation preventing stress induced ATF4 expression. Cells containing empty vector (EV), expressing wild type eIF2α (WT), or phosphorylation-defective eIF2αS51 (KI) were tested for their capacity to phosphorylate eIF2α in response to stress, 0.1μM Thapsigargin (TG) for 6h, by western blot using anti-phosphorylated eIF2α antibody. TG was used to induce ER stress to promote eIF2α phosphorylation. In the EV and WT cell lines, this stress increased eIF2α phosphorylation concomitant with ATF4 induction. In the eIF2α mutant cell line (KI) eIF2α phosphorylation and ATF4 was substantially inhibited. The images represent three independent experiments. β-Actin was used as a loading control.
Mean Fold Change in Sphere Formation (log_2)

Fold Change in Gene Expression in Hypoxia Relative to Normoxia

a

b

Normoxia Hypoxia

EV WT KI

WT

KI

Snail Snail Twist Twist Vim Vim ZEB ZEB

WT KI WT KI WT KI WT KI
Figure 4.5: Phosphorylation-Deficient eIF2α Mutants Demonstrated Impairment in Tumoursphere Formation in Response to Hypoxic Stress.

Single cells were seeded at 1 cell per well in a 96 well non-adherent plate. (a) Both empty vector (EV) and wildtype eIF2α (WT) MDA-MB-231 cells increased their respective rates of tumoursphere formation in response to hypoxia (0.5% O₂, 24h) relative to their normoxic (20% O₂) controls (p<0.05). Cells with the S51A mutant eIF2α (KI) had lower sphere formation rates in hypoxia than they did in normoxia (p<0.05, n=3). The eIF2α (KI) line, when treated with hypoxia, showed reduced sphere formation capacity compared to EV and WT in the same conditions. Letters indicate a significant difference determined by one-way ANOVA. Bars are the mean fold change of tumourspheres ± SD. (b) Images are representative of the spheres formed by each treatment group (500µm scale bar). (c) Real time RT-PCR analysis of MDA-MB-231 WT and KI cell lines for SNAIL, TWIST, VIM, TWIST1, and ZEB1. MDA-MB-231 cells were cultured in 20% or 0.5% oxygen for 24h. Hypoxia samples were normalized to paired normoxia samples and represented as log2 fold change (n=3). TWIST1, VIM and ZEB1 increased in hypoxia in the eIF2α (WT) MDA-MB-231 line and that increase in prevented in eIF2α (KI) MDA-MB-231 line significant differences are indicated by the asterisks (*). All bars represent ± SD relative to the 20% O₂ control. Significance was determined using paired Student’s t-tests.
4.1.2 The ISR helps Protect Against the Chemotherapeutic Agent Paclitaxel.

Cancer stem cells and plasticity are implicated as significant factors in chemoresistance [568]. ALDHhiCD44+ breast cancer stem cells possess an ALDH-dependent form of chemoresistance [569]. Evidence is also accumulating that proteins regulating translation, like PERK, act as central nodes regulating a multitude of chemoresistance pathways [570]. We tested the hypothesis that preventing the translational response to limit plastic adaptation and stress pathway activation would increase the breast cancer cells sensitivity to chemotherapy. To do this, cells were treated with paclitaxel ranging from 2.5nM to 50nM in the presence or absence of ISRIB (Fig. 4.6). We tested the effects of ISRIB on chemoresistance in two breast cancer cell lines — SUM149 (Fig. 4.6 a) and T47D (Fig. 4.6 b). ISRIB increased the sensitivity of the cells by as much as 5-fold in the T47D cells, measured as Coomassie blue stained colonies after five days (p<0.05, n=3). ISRIB impairs chemoresistance signalling in cell culture at a broad range of concentrations depending on the cell line tested.

4.1.3 The Effects of Chemotherapeutic Stress on In Vivo Cancer Stem Cells and Tumour Development

We sought to verify these findings in an in vivo system to determine if ISRIB can increase the efficacy of paclitaxel with an intact tumour microenvironment. Six mice bearing bilateral SUM149 tumours were assigned to each treatment group; DMSO control, ISRIB (2.5mg/kg) paclitaxel (20mg/kg), and the combination therapy of ISRIB and paclitaxel (ISRIB+Pac) (Fig. 4.7). ISRIB and paclitaxel were dosed every second day for two weeks by IP injection starting when the tumour diameter reached 5mm. By day twelve, all treatment groups showed a significant decrease in size relative to the control group. By day eighteen all groups had returned to control levels except for the combination therapy (one-way ANOVA p<0.01, n=12). In this model, ISRIB enhances the effects of paclitaxel when paclitaxel slows tumour growth but does not eradicate the tumour. This decrease in size resulted in a significant increase in the survival of the group receiving the
Figure 4.6: Inhibition of the ISR by ISRIB Decreases In Vitro Chemoresistance to Paclitaxel.

(a) Viability assay wherein SUM149 cells were plated into six-well plates, allowed to attach, treated, then grown for 5 days to facilitate visible colony formation. Paclitaxel was dosed at 5nM and 10nM for 3 hours with or without ISRIB (10nM) as normalized to vehicle treated controls. Cells exposed to the combination therapy, paclitaxel and ISRIB, induced greater cell death than paclitaxel alone (p<0.05, n=3). (b) Dose-response viability assay for colony formation. Cells received doses of paclitaxel ranging from 0.25nM to 5nM for 3 hours with or without ISRIB (10nM). Cells exposed to the combination therapy, paclitaxel and ISRIB, were killed more than those treated with paclitaxel alone. Doses of paclitaxel improved by ISRIB (10nM) ranged from 2.5nM to 50nM (p<0.05, n=3). Horizontal lines indicate significant differences using Student’s t-test. Bars are the mean percent survival as measured by mean CFU ± SD.
Figure 4.7: Inhibition of the ISR In vivo Retards Growth of SUM149 Tumours Treated with Paclitaxel.

DMSO control, ISRB (2.5mg/kg) paclitaxel (20mg/kg), and the combination therapy of ISRB and paclitaxel (ISRB+Pac) were administered to NSG mice bearing bilateral SUM149 tumours every second day beginning at a tumour diameter of 5mm and continued for two weeks. Box and whisker plots showing the distribution of tumour size in each group. Growth retardation occurred in the ISRB and the combination groups, as measured by tumour diameter along the longest dimension at 12 days (one-way ANOVA p<0.01, n=12). At 18 days the combination therapy outperformed all other groups in the experimental set. Circles (○) mark individual tumours, and statistical differences, as determined by one-way ANOVA, are indicated by horizontal lines.
Figure 4.8: Kaplan-Meier Plots of Tumour Bearing Mice Receiving Paclitaxel and ISRIB Treatments.

Survival of NSG mice from DMSO, ISRIB (2.5mg/kg), paclitaxel (20mg/kg), and the combination therapy (ISRIB and paclitaxel) treatment groups. NSG mice treated with ISRIB and Paclitaxel showed significantly higher survival than control DMSO treated mice (p=0.0034, n=6). Mice were sacrificed when tumour diameter reached 10mm. The probability of survival curves were calculated using the Kaplan-Meier product-limit method and compared via the log-rank test between control and treatment groups.
combination therapy (p=0.0034, n=6) (Fig. 4.8). The sensitivity of tumours derived from the SUM149 cell line to cytotoxic chemotherapy was increased by coadministration with ISRIB.

From these tumours, we extracted a single cell suspensions and tested them for their ability to form spheres in non-adherent plates (Fig. 4.9). The administration of ISRIB decreased the sphere forming capacity of the cells by more than 70% (p<0.001, Control n=11, ISRIB n=11, Pac n=10, ISRIB+Pac n=12) (Fig. 4.9 a). Neither the increase in sphere formation from paclitaxel nor the return to baseline in the combination group was statistically significant. The microphotographs in Figure 4.9 b are representative images of spheres derived from cells in each treatment group. Having observed that ISRIB is capable of inhibiting sphere formation in vivo, we then tested to see if there was a simultaneous suppression of the ISR stress response. Mirroring the observed reliance of ATF4 expression on eIF2α phosphorylation that we observed in vitro (Fig. 4.4), we utilized IHC to determine if ISRIB decreased the ATF4 expression in tumour tissue sections in vivo. In addition, using ATF4 as an output for ISR we can use its expression to determine if paclitaxel induces an ISR stress response. Tumour sections from the SUM149 tumours were stained with an anti-ATF4 antibody, and ATF4 expression was quantified by counting the number of strongly positive cells in each field of view for three fields of view in four tumours (Fig. 4.10). The representative images are of fields of view used to quantify ATF4 staining in each treatment group are presented. We observed a significant increase in the number of high-ATF4-expressing cells in tumours treated with paclitaxel (p<0.01, n=4) (Fig. 4.4 a). Treating the tumours with ISRIB in addition to paclitaxel causes a full reversion of this phenotype. The decreased expression of ATF4 is both a valuable positive control that demonstrates ISRIB’s capacity to affect signalling in vivo and an experimental variable for evaluating how the administration of different treatments is altering ISR.

Next, we sought to determine if eIF2α phosphorylation the activation of the ISR would increase tumour colonization in the lung using a tail-vein lung metastasis assay. This assay is similar to a limiting dilution assay, but the tail vein injection requires multiple steps in the metastatic cascade including travel through the vasculature, extravasation and
Figure 4.9: Assessing the Effects of ISRIB on Treatment-based Increases of Cancer-Stem-Cells.

Sphere formation assay for cells extracted from DMSO, paclitaxel (20mg/kg), ISRIB (2.5mg/kg) and the combination therapy (ISRIB and paclitaxel) treated tumours. Filtered and counted single cell suspensions from dissociated 10mm tumours were seeded at 100 cells were per well in a 96 well non-adherent plate. The treatment of SUM149 tumours by ISRIB (2.5mg/kg) results in fewer CSCs as measured by sphere formation (p<0.001). Intraperitoneal paclitaxel (Pac, 20mg/kg) did not significantly increase sphere formation, nor was ISRIB sufficient to decrease sphere formation when administered in combination (Control n=11, ISRIB n=11, Pac n=10, ISRIB+Pac n=12). An asterisk (*) indicates significant differences from all other groups by one-way ANOVA. Bars are the mean number of tumourspheres ± SD. (b) Images are representative of the spheres formed by each treatment group (250µm scale bar).
Figure 4.10: In Vivo ATF4 Expression Resulting from Chemotherapy Induced Stress is Reduced by ISRIB.

(a) Quantification of IHC staining for ATF4 expressing cells in SUM149 tumours from mice treated with DMSO, ISRIB (2.5mg/kg), paclitaxel (Pac, 20mg/kg), or ISRIB and paclitaxel (ISRIB+Pac). Paclitaxel increases the amount of ATF4 high cells, but when used in combination with ISRIB the number of ATF4 expressing cells returns to control levels. The asterisk (*) indicates a significant difference from all other groups by ANOVA. Bars are the mean number of ATF4 positive cells ± SD. (b) Images are representative of 1 field of view for three independent experiments. Brown nuclear staining defines ATF4 expression. Three random fields of view were counted for each tumour (p<0.01, n=4).
seeding and outgrowth at secondary sites. To test the role of stress in priming cells for metastasis, SUM149 cells were pretreated with DMSO vehicle control or Salubrinal (10µM) for 24h before 500,000 cells in 700µL Ca2+-free HBSS were injected into the tail vein of mice. The mice were sacrificed at eight weeks, and Anti-Human HLA was used to detect tumour cells in the lung sections (Fig. 4.11 c). The total number of metastases per section were counted for ten mice (Fig. 4.11 a). On average, mice that received tumour cells treated with Salubrinal had double the number of lung metastases (Fig. 4.11 a) and were more than twice as likely to have any tumour in the lungs (Fig. 4.11 b). Representative images of lung metastases can be seen in Figure 4.11 c. These data suggest that intermediate stress resulting in the activation of the ISR and the subsequent alterations in translation may prime cells to more successfully metastasize.

If ISRIB reduces the tumour’s inherent tolerance to stress it may alter the structure of the tumour as it grows, consequently changing the distribution of cells found within the necrotic regions of the tumour. Tumour sections from DMSO control (n=9), INK128 (INK, 30mg/kg, n=12), ISRIB (2.5mg/kg, n=12), ISRIB and INK128 (ISRIB+INK, n=10), paclitaxel (Pac, 20mg/kg, n=10), or ISRIB and paclitaxel (ISRIB+Pac, n=9) were H&E stained (Fig. 4.12). From these slides, we determined the cellular (nuclei positive fraction) within the necrotic area as a subset of the total tumour mass. The results are presented as the percent of the total tumour composed of cells within the necrotic regions (nuclei positive necrotic fraction). Most of the results are described in chapter 3 (Fig. 3.14). Like ISRIB, the combination of ISRIB and paclitaxel decreases the amount of necrosis that the cells can tolerate. There is a large body of evidence that links stress to tumour progression, so it is possible that this decrease in cells in the necrotic regions of the tumour may be one of the contributing factors to the increased survival of the cohort receiving the combination therapy seen in Figure 4.8 [571, 572].

Animal studies performed in a single model are difficult to generalize as the experimental variables are being measured against a single genetic background. The use of cell lines further limits the ability to generalize one's findings to broader populations as cell lines do not necessarily reflect the full mutational and transcriptional profiles of the cancers from which they were derived [573, 574]. To help address these limitations we test two
Figure 4.11: Salubrinal Alters the Dynamics of Metastasis to the Lungs.

Tail vein lung metastasis assay to address the potential of UPR stress to affect metastasis. (a) 500,000 SUM149 cells or SUM149 cells treated with Salubrinal (10µM) for 24h, in 700 µL Ca2+-free HBSS, were intravenously injected into the tail vein of NSG mice. The lungs were sectioned and stained with human HLA to detect the presence of any human tumour cells at 8 weeks. Salubrinal increases seeding in the lungs (p=0.042, t-test, n=10). The asterisk (*) indicates a significant difference. Individual mice are marked by circles (○). (b) Percentage of mice with detectable micrometastases in the non-treatment and treatment group. (c) 20x bright field microscope images of micrometastases formed in each treatment group.
% Cellular Fraction Necrotic Regions in the Tumour

DMSO  INK  ISRIB  ISRIB+INK  Pac  ISRIB+Pac
Figure 4.12: Quantification of Hypoxic Regions in Tumours Treated With INK, ISRIB, ISRIB and INK, Paclitaxel, and ISRIB and Paclitaxel in combination.

ISRIB and ISRIB in combination with paclitaxel decrease the cellularity of necrotic areas. Percent of the necrotic area that contains cells from hematoxylin and eosin (H&E) stained SUM149 tumour sections from six treatment groups; DMSO control, INK128 (30mg/kg), ISRIB (2.5mg/kg), or ISRIB and INK128 (ISRIB+INK), paclitaxel (Pac, 20mg/ml), and ISRIB and paclitaxel (ISRIB+Pac) was measured using ImageJ and is represented as percentage of the total tumour area. Total tumor section area was selected and subsequently measured by thresholding against background pixel intensity. Second round of thresholding against lighter necrotic tumor regions was used to measure total area of necrosis. Cellular necrotic regions were determined by dense dark areas within necrotic regions, where cells have intact nuclei. All areas were measured in pixels and all threshold selections were manually examined during analysis to ensure correct assignment of necrotic or cellular necrotic regions. Pictures are representative images of tumours from each treatment group. Statistical significance was determined by Student’s t-test (p<0.05).
PDX models — PDX401 and PDX574, in addition to the SUM149 cell line. PDXs have been shown to robustly reproduce the mutational profile of tumours while maintaining other fundamental histological characteristics of the source tumour [575-577]. Two mice bearing bilateral PDX401 tumours were administered DMSO, ISRIB (2.5mg/kg) paclitaxel (Pac, 20mg/kg), or the combination therapy (ISRIB+Pac) by intraperitoneal injection every second day for two weeks beginning when the tumours reached a diameter of 5mm across their longest dimension (Fig. 4.13). Upon completion of this regimen, tumours were allowed to grow to 10mm in diameter before the tumours were extracted and analyzed. Only ISRIB showed a statistically significant reduction in tumour size compared to DMSO and ISRIB+Pac (p<0.05, n=4). Though the ISRIB mice did survive longer than the other groups with only two mice, no conclusions can be drawn from the result.

We tested for changes in plasticity by extracting cells from the tumours, enumerating them, and plating them at a density of 100 cells per well in 96 well non-adherent plates, and finally assessing the rate of sphere formation (Fig. 4.14 a). The PDX401 tumours that were treated with paclitaxel demonstrated a marked increase in sphere formation whereas ISRIB used as a monotherapy reduced the sphere formation by ~33%. The most substantial change occurred when ISRIB and paclitaxel were used in combination. In this treatment group, there was less than 5% of the sphere forming capacity observed in the paclitaxel group. Representative images of spheres formed from cells extracted from tumours in each treatment group can be seen in Figure 4.14 b. Under this dosing regimen, ISRIB used in combination with paclitaxel appears to have a complementary effect that targets cells with sphere-forming capacity.

The second PDX model, PDX574 (Fig. 3.18), is derived from a more aggressive tumour that has higher levels of both hypoxia, as determined by the CA9 expression, and NODAL expression. This model, in conjunction with SUM149 and PDX401, represents a broad range of phenotypic diversity that should better represent the natural breast cancer population than any one model or cell line could. We administered the same treatments as the previous two test groups; DMSO, ISRIB (2.5mg/kg), paclitaxel (Pac, 20mg/kg),
Four different treatments were administered to NSG mice bearing bilateral PDX401 tumours — DMSO, ISRIB (2.5mg/kg) paclitaxel (20mg/kg), and a combination therapy (ISRIB+Pac). Mice were treated every second day for two weeks beginning when a tumour reached a diameter of 5mm across its longest dimension. Box and whisker plots showing the distribution of tumour size (length of the longest axis) in each group. Growth retardation occurred in the ISRIB treatment group exclusively (p<0.05, n=4). At 14 days the combination therapy failed to outperform the control group, and the tumours were statistically larger than the ISRIB group. Circles (o) mark individual tumours, and statistical differences are indicated by horizontal lines. One-way ANOVA was used to determine statistical significance.
Figure 4.14: ISRIB Decreases Cancer-Stem-Cells and Chemotherapy Induced CSCs In PDX401 Tumours.

Sphere formation assay for cells extracted from PDX401 tumour. A suspension of single cells from dissociated PDX401 tumours from NSG mice treated with DMSO, ISRIB (2.5mg/kg) paclitaxel (20mg/kg), or a combination therapy (ISRIB+Pac) was seeded at 100 cells per well in a 96 well non-adherent plate. (a) Intraperitoneal paclitaxel (20 mg/kg) increased the abundance of CSCs as measured by the frequency of sphere formation from PDX401 tumours. ISRIB (2.5mg/kg) and ISRIB with paclitaxel resulted in fewer CSCs (n=4). All groups were significantly different from one another by one-way ANOVA (p<0.001). Letters indicate significant differences. Bars are the mean number of tumourspheres ± SD. (b) Images are representative of the spheres formed by each treatment group (500µm scale bar).
Figure 4.15: Growth and Kaplan-Meier Plots Demonstrating Increased Survival of Tumour Bearing Mice Receiving Paclitaxel and ISRIB Treatments.

Upon PDX574 mammary fat tumours implanted into NSG mice reaching a diameter of 5mm, mice were administered DMSO, ISRIB (2.5mg/kg), paclitaxel (20mg/kg), or ISRIB and paclitaxel (ISRIB+Pac) every second day for two weeks. (a) There was no significant change in tumour size, measured by calipers along the longest axis. Significance determined by one-way ANOVA (n=6). Individual tumours are marked by circles (○). (b) Only the combination of ISRIB and paclitaxel demonstrated enhanced survival as compared to DMSO (p<0.01, n=6). The probability of survival curves were calculated using the Kaplan-Meier product-limit method and analyzed via the log-rank test between treatment groups.
or ISRIB or paclitaxel (ISRIB+Pac) every second day for two weeks beginning at 5mm tumour diameter (Fig. 4.15 a). There was no difference in size between any of the treatment groups as determined by caliper measurements. When survival was measured and tested by log-rank test, the combination group demonstrated superior survival than any of the other three groups (Fig. 4.15 b). We again observe, this time in a more advanced model of breast cancer, the survival benefits of combining ISRIB and paclitaxel.

The route of drug administration affects its bioavailability [578]. Though IP injections of paclitaxel are commonly used in animal studies of cancer as a means of administering the drug efficiently and safely to animals, we suspected that this might result in decreased efficacy of paclitaxel. To address these concerns, we tested weekly intravenous paclitaxel injections of 20mg/kg for two weeks in combination with orally administered ISRIB (10mg/kg daily for three weeks). With this dosing strategy, we treated mice bearing bilateral PDX401 (Fig. 4.16 a) and PDX574 (Fig. 4.16 b) tumours starting at 5mm. PDX401 tumours respond better to IV paclitaxel than they did to IP paclitaxel (Fig. 4.16 a and Fig. 4.13). Paclitaxel and the combination therapy (ISRIB+Pac) caused a substantial decrease in tumour size. The reductions in tumour size resultant from the two therapies were significant from both the control and the ISRIB group (p<0.01). This result contrasts with PDX574 (Fig. 4.16 b), which demonstrated resistance to paclitaxel regardless of the method of administration (Fig. 4.16 b and Fig. 4.13). In both models — PDX401 (Fig. 4.17 a) and PDX574 (Fig. 4.17 b) — paclitaxel caused an increase in survival relative to controls (significance could not be achieved due to censorship of 2 mice in PDX401). Though there was an increase in the survival of ISRIB-treated mice in both PDX models, the sizes of the experimental cohorts (PDX401 n=4 and PDX574 n=4) was insufficient to rule out the possibility of this being the result of chance. ISRIB did not enhance the survival benefits of paclitaxel in either model.

We and others have shown ISRIB alters stress responses that are coordinated through the ISR. This stress response occurs concomitantly with alterations to the transcriptome and translatome that increase plasticity. Using sphere formation assays, we tested
Figure 4.16: IV Paclitaxel, ISRIB and Their Combined Use are Effective at Controlling Tumour Size in PDX401 but not PDX574.

Box and whisker plots for PDX401 (a) and PDX574 (b). Upon tumour diameter reaching 5mm, the mice were administered DMSO (n=8), ISRIB (10mg/kg daily for 3 weeks) (n=8), paclitaxel (20mg/kg weekly for 2 weeks) (n=8), or ISRIB and paclitaxel (ISRIB+Pac) (n=6). (a) Additional days beyond the first twelve are shown to illustrate the rate and distribution of the recovering PDX401 tumours after treatment withdrawal. Some mice were censored due to unrelated complications. Tumour sizes is the length of the longest dimension of the tumour. Changes in size are observable as early as 5 days after treatment initiation in PDX401 (p<0.01) (a). There was no observed change in the rate of growth or tumour size in PDX574 (n=6) (b). Individual tumours are marked by circles (○) and horizontal lines indicate significant differences by one-way ANOVA.
Figure 4.17: Kaplan-Meier Plots of Two of Different PDX Tumour Models in Mice Receiving Paclitaxel and ISRIB Treatments.

Kaplan-Meier Curves for the tumours in Figure 4.16 (a) NSG mice bearing PDX401 tumours that received intravenous paclitaxel (20mg/kg weekly for 2 weeks), ISRIB (10mg/kg daily for 3 weeks), or ISRIB and paclitaxel (ISRIB+Pac) showed significantly higher survival than DMSO treated mice (p<0.01, n=4). (b) PDX547 tumours, from NSG mice receiving the same treatments as in (a), and showed greater survival in mice receiving paclitaxel, or ISRIB and paclitaxel (ISRIB+Pac) as compared to DMSO treated mice (p<0.01, n=4). The probability of survival curves were calculated using the Kaplan-Meier product-limit method and compared via the log-rank test between control and treatment groups.
whether stress induced by paclitaxel increased sphere formation and if coadministration with ISRIB can protect against increases in plasticity. For both PDX models tumours from each treatment group, DMSO as a control, ISRIB (10mg/kg daily for three weeks), intravenous paclitaxel (20mg/kg weekly for two weeks), or ISRIB and paclitaxel (ISRIB+Pac), were processed, single cells extracted and then seeded at 100 cells per well in a 96 well non-adherent plate. PDX401 tumours treated with ISRIB as a monotherapy demonstrated a 50% sphere formation; paclitaxel increased sphere formation by ~30%; ISRIB used in combination with paclitaxel reduced sphere formation below that of the control (DMSO) to the level of the ISRIB monotherapy (Fig. 4.18 a, p<0.001, n=6).

Images in Figure 4.18 b show representative examples of spheres formed from tumours in each treatment group. In PDX574, paclitaxel induced a significant increase in sphere formation. ISRIB treatment alone did not cause a significant reduction in sphere formation but when used in combination with paclitaxel the increase in sphere formation was abrogated (Fig. 4.18 c, p<0.01, n=6). Images in Figure 4.18 d show representative examples of spheres formed from tumours in each treatment group. In two different clinically relevant PDX models representing a broad range of endogenous hypoxia levels we show that chemotherapeutic stress increases phenotypic plasticity and that this effect can be reversed by preventing ISR signalling with ISRIB. In the highly metastatic MDA-MB-231 cell line both tumour size and sphere formation improved when promoted by ISRIB coadministered with paclitaxel (Supplemental Figure 12).

To confirm that ISRIB is altering signalling in the tumours, we employ IHC to measure ATF4 with an Anti-ATF4 antibody. ATF4 expression was quantified by counting the strongly positive cells (dark brown staining) in each field of view for three fields-of-view in four tumours (Fig. 4.19 a). ISRIB decreased the number of positive cells compared to control groups whereas paclitaxel increased them. ISRIB administered in combination with paclitaxel reduced the ATF4 level to that of the DMSO controls but was insufficient to reduce the ATF4 expression to that of the ISRIB monotherapy. The images in Figure 4.19b show representative examples of ATF4 stained tumour sections from each treatment group. In the PDX574 tumours, the basal level of ATF4 expression is high as would be expected in a tumour with high levels of hypoxia. ISRIB significantly decreased the expression of ATF4 in these tumours (Supplemental Figure 13).
Figure 4.18: ISRIB Decreases Cancer-Stem-Sells In PDX401 and PDX574 Tumours.

Tumoursphere formation assay for cells extracted from fresh PDX401 and PDX574 tumours. The mice received DMSO as a control, ISRIB (10mg/kg daily for 3 weeks), intravenous paclitaxel (20mg/kg weekly for 2 weeks), or ISRIB and paclitaxel (ISRIB+Pac). Single cell extracts were seeded at 100 cells per well in a 96 well non-adherent plate. (a) In mice with PDX401 tumours, IV paclitaxel (20mg/kg) increased the abundance of CSCs as demonstrated phenotypically as an increase in the frequency of sphere formation. ISRIB (10mg/kg) reduced levels of sphere formation below control levels, and ISRIB with paclitaxel also diminishes tumoursphere formation below control levels (p<0.001, n=6). (b) Images are representative of the spheres formed by each treatment group from each PDX model (250µm scale bar). (c) In the PDX574 group ISRIB did not decrease sphere formation, but was sufficient to do so in cells treated with paclitaxel (p<0.01, n=6). Letters indicate significant differences from other treatment groups as tested by one-way ANOVA. Bars are the mean number of tumourspheres ± SD. (d) Images are representative of the spheres formed by each treatment group from each PDX model (250µm scale bar).
a

Number of ATF4 Positive Cells

DMSO ISRIB Pac ISRIB+Pac

PDX401

b

DMSO ISRIB Pac ISRIB+Pac

PDX574
Figure 4.19: ATF4 Expression Induced by Chemotherapy is Reduced by ISRIB In Vivo.

(a) Quantification of cells expressing high levels of ATF4 in PDX401 tumours from mice receiving either DMSO, 10mg/kg ISRIB, 20mg/kg interavenous paclitaxel (Pac), or ISRIB and paclitaxel (ISRIB+Pac) as assessed by IHC. ISRIB reduced the number of cells expressing ATF4, and paclitaxel increased ATF4 expressing cells relative to control. ISRIB, when used in combination with paclitaxel, returned the number of ATF4 expressing cells to that found in the DMSO control. Horizontal lines indicate significant a relationship between groups as determined by one-way ANOVA. Bars are the mean number of ATF4 positive cells ± SD. Pictures are representative of 1 field of view for three independent experiments. Brown nuclear staining defines ATF4 expression. Three random fields of view containing viable tumour tissue were counted for each tumour (p<0.05, n=3). (c) Images of IHC staining for PDX574 tumour sections containing viable tumour tissue from NSG mice probed for ATF4 expression (brown) receiving either DMSO, or 10mg/kg ISRIB. ISRIB reduced the amount of ATF4 expression. Pictures are representative of the staining found in PDX574 tumours.
Figure 4.20: The Effect of IV Paclitaxel and IV Paclitaxel in Combination with ISRIB on MDA-MB-231 Tumour Growth and CSC Frequency.

(a) Box and whisker plot for MDA-MB-231 tumour volume in NSG mice 7 days after the final treatment dose. Upon tumour diameter reaching 5mm, the mice were administered paclitaxel (IV 20mg/kg weekly for 2 weeks) (n=6), or ISRIB (2.5mg/kg IP every second day for 2 weeks) and paclitaxel (ISRIB+Pac) (n=6). (a) Tumour size is the length of the longest dimension of the tumour. Tumours receiving the combination treatment demonstrated reduced tumour growth (p<0.01). (b) Tumoursphere formation assay for cells extracted from fresh MDA-MB-231 tumours. The mice received intravenous paclitaxel (Pac) or ISRIB and paclitaxel (ISRIB+Pac) as above. Single cell extracts were seeded at 100 cells per well in a 96 well non-adherent plate. ISRIB decreased the abundance of CSCs compared to tumours receiving only paclitaxel. The bars signify the mean fold change in sphere formation ± SD (p<0.05, n=6). Images are representative of the spheres formed by each treatment group from each PDX model (250µm scale bar). Significance was determined using Student’s t-test. Asterisks (*) indicate a significant difference between treatment condition.
Figure 4.21: Analysis of RNA-seq from 1100 Breast Cancer Patients

Demonstrating Dysregulation of PERK and eIF2α (TCGA) and the Prognostic Value of *PERK*.

(a) Expression of *PERK* (*EIF2αK3*) transcript increases relative to normal adjacent breast tissue. The target of PERK’s kinase activity, eIF2α, decreases in tumours relative to normal adjacent breast tissue. The abundance of both transcripts was normalized and estimated using RSEM (Student’s t-test, p<0.001). (b) A Kaplan-Meier plot demonstrating the correlation between PERK expression (top 50% of expressers versus the bottom 50%) and survival. Low PERK expression is predictive of survival for 19 years in these data. (c) The hazard ratio demonstrates a significant risk to patients with higher levels of *PERK* compared to their low expressing cohort (Wilcoxon, p=0.00108). Low EIF2AK3 (n=479), High EIF2AK3 (n=479).
We also tested whether ISRIB could improve the efficacy of paclitaxel in the dedifferentiated highly metastatic breast cancer cell line MDA-MB-231, using a two-arm study design. Seven days after the final IV paclitaxel dose, tumours in the group receiving the combination therapy showed statistically significant growth retardation compared to the paclitaxel group (Fig. 4.20 a). As with our previous experiments, ISRIB reduced the frequency of CSCs as measured by sphere formation (Fig. 4.20 b).

To assist in determining if inhibiting stress-induced ISR signalling could help patients, we analyzed RNA-seq data from 1100 breast cancer patients’ tumours and compared the expression of PERK and eIF2α to that of normal adjacent breast tissue. In these samples PERK, the inhibitor of eIF2α, is upregulated in breast cancer and eIF2α is considerably downregulated (Fig. 4.21 a). Alterations in the balance of effector and target of this type should favour stronger translational inhibition supporting the concept that increased stress response is an unfavourable trait in tumours. This hypothesis is further supported by the survival data of patients dichotomized by median PERK expression. Patients with lower levels of PERK survive at a significantly higher rate than those with higher levels of expression for 15 years following diagnosis (Fig. 4.21 b). The clinical evidence from patient data supports the hypothesis that the capability for stress adaptation correlates with both tumourigenesis and survival.

4.2 Discussion

Stress pathways often have two distinct roles in cellular physiology. The first is that they are to be protective, insulating the cells from environmental harm. The second is when stress occurs, for either a sufficient duration or severity, these pathways can induce apoptosis. In a review titled *Apoptosis and Autophagy: regulatory connections between two supposedly different processes*, Dr. Andrew Thorburn describes the rationale for thinking about autophagy and apoptosis as a continuum rather than separate pathways [579]. So too can we think of hypoxia and HIF1α as a continuum [580]. It is clear from research on the tumour microenvironment and apoptosis that stress and cell death are consistently part of the tumour milieu and drive disease progression [581-583]. However, because of the spectrum of response that can arise from stress, how we model cancer must be carefully considered to address which aspect of the adaptation-apoptosis
spectrum we want to study. As hypoxia correlates with poor prognosis, we hypothesized that induction of the ISR, like that seen in hypoxia, would result in pro-tumourigenic changes to plasticity. We sought to investigate the role of sub-apoptotic stress to address how those stresses stimulate changes that alter plasticity. This approach allows us to understand better what is occurring in the surviving fraction of tumour cells under adverse conditions [479, 507, 571].

In hypoxia, the activation of PERK to suppress global mRNA translation is a vital adaptation, and the subsequent diminution of eIF2α activity is a crucial mediator of translational activity in times of stress. ISR is a series of pathways that converge to signal through eIF2α, which ultimately leads to the expression of factors like ATF4 to elicit a stress response [570]. There is no better example of the complexity and balance of these systems than the proto-oncogene c-Myc. c-Myc increases proliferation or apoptosis based on cellular context [584, 585]. The proliferative effects of c-Myc are at least in part the result of the higher protein synthesis that results from increases in ribosome biogenesis [586]. This elevated protein synthesis, in turn, requires increased PERK expression and activation of PERK-eIF2α-ATF4 to sustain the amount of ER stress induced by the elevated levels of proliferation [587]. Here we attempt to elucidate further the mechanisms by which PERK and eIF2α contribute to tumourigenesis by investigating their role in the emerging concept of stress-induced plasticity.

Emerging data is validating the concept that stress can induce plasticity resulting in a stem-like state and that among these stresses are both hypoxia and chemotherapy [236, 458, 588, 589]. We first demonstrate this by using a proline analogue (AZE) to prevent proper protein folding and induced UPR stress that increased NODAL expression, which we have previously shown to be important in stress-induced plasticity (Fig. 4.1, Fig. 2.1). We confirm these results using a second small molecule to mimic the PERK arem of the UPR response, Salubrinal. The administration of this chemical also increases NODAL expression. Expectedly, ISRIB, which mitigates the effect of eIF2α phosphorylation, decreased NODAL protein expression (Fig 4.2). Our previous evaluations of plasticity have indicated that changes result from a network of proteins rather than a single driver, so we measured the levels of NANOG, SOX2, SNAIL, and SLUG, all of which were
upregulated by Salubrinal (Fig 4.3). Changes in SNAIL and SLUG that result from ER stress likely point to a bidirectional relationship between the pathways that ER stress induces and EMT. The gene expression that is associated with EMT may help stress adaptation, but EMT increases the strain on the ER to support the secretion of additional extracellular proteins [410].

Two approaches could have been explored to evaluate the potential for therapeutic mitigation of this pathway. The first was to use a PERK inhibitor. This approach was not pursued because PERK inhibitors, though capable of reducing tumour dissemination, lead to pancreatic atrophy and thus have limited clinical potential. The second option was to evaluate the role of eIF2α for which there is a promising and well-tolerated inhibitor (ISRIB) [590]. This strategy can also potentially address the ISR signalling that does not exclusively signal through PERK, like that of amino acid starvation, nutrient deprivation, or even oncogenic transformation [549, 587, 591]. Using the MDA-MB-231 KI cell line, we demonstrated that the induction of sphere formation observed, which resulted from exposure to hypoxia, was dependent on the phosphorylation of eIF2α (Fig. 4.4, Fig. 4.5). We then demonstrated that several genes could contribute to these phenotypes, namely TWIST, VIM, and ZEB are no longer upregulated. The effects of eIF2α phosphorylation on translation, though potentially significant for understanding alterations in plasticity, have not been evaluated.

Many anti-cancer therapies result in the induction of the UPR, likely to address the resulting therapeutic stress (Fig 2.12) [410, 592]. It is also true that the activation of PERK and the subsequent phosphorylation of eIF2α can confer drug resistance [429, 593, 594]. For this reason, inhibition of the adaptive response to stress that ultimately results in the phosphorylation of eIF2α may sensitize breast cancer to radio and chemotherapies. We demonstrated in two different poorly aggressive breast cancer cell lines that ISRIB could sensitize cancer cells to paclitaxel. These types of preliminary findings increase the plausibility of either making therapies more effective or by decreasing the dose necessary to treat the disease and thus minimizing the side effects experienced by patients, though much more work must be done to determine the potential of ISRIB in the clinic.
For our animal experiments, we tested ISRIB, paclitaxel and the combination therapy (ISRIB+Pac) against the control arm. This set-up allowed us to observe how the inhibition of the ISR differed from the effects of cytotoxic chemotherapy and begin to understand how these treatments may be used together. There is a practical reason for including first line chemotherapy in preclinical experimental design. Many current cancer clinical trials fall into those with an active comparator or those against historical data. In both cases, the clinical data is being compared, in essence, to that of the current standard of care [595]. For these reasons, we included a paclitaxel and an ISRIB and paclitaxel arm in each of our studies.

We tested the effects of ISRIB in several models and with two different dosing regimens. We compared the control placebo (DMSO) and paclitaxel (the standard of care) to ISRIB, and paclitaxel co-administered with ISRIB (ISRIB+Pac). Responses varied depending on the mode of administration and the characteristics of each model tested. The first experiment was performed in mice bearing SUM149 tumours, which were receiving what we determined to be a sub-optimal dosing strategy for paclitaxel (Fig. 4.7). SUM149 cells respond to IP ISRIB as a monotherapy. With this administration where paclitaxel is less bioavailable, it was observed that ISRIB enhances its efficacy with regard to both tumour size and survival (Fig. 4.7 and Fig 4.8). It is hard to derive a definitive interpretation of these results, but it does suggest that there may be a yet undefined subset of patients for which the combination therapy is appropriate.

In cells extracted from these tumours, we observe a decrease in the sphere formation capacity with the administration of ISRIB. Paclitaxel delivered by IP did not significantly increase the sphere forming capacity of these tumours. Having tested sphere formation, we assessed the activation of the ISR with ATF4 using IHC. We confirm that paclitaxel treatment activates the ISR and that ISRIB can mitigate the ATF4 portion of this signalling. Having previously established that stress, namely hypoxia, can restrict translation thereby enhancing the expression of stress-related genes like ATF4 and those that regulate plasticity, and that paclitaxel induces a stress that signals through the same pathways, we think this is strong evidence that there is an in vivo translational component to the phenomena we are observing. However, because there are very few papers that
directly address translation in response to chemotherapy, the proportion of the response that is the result of translation has yet to be determined [596]. The hypothesis that inhibition of the ISR would decrease the tolerance to stress fits with the observation that ISRIB, and ISRIB in combination with paclitaxel both decrease the total area of hypoxia found in the tumours derived from SUM149 cells (Fig. 4.12).

Using SUM149 cells, we demonstrated that eIF2α phosphorylation increases the seeding of cells in the lung (Fig. 4.11). These data suggest the potential of stress at the primary site to prime cells to more successfully metastasize.

We tested two PDX models and in each we utilized two modes of administration for each drug; PI ISRIB with PI paclitaxel, and gavage ISRIB and IV paclitaxel (Fig. 4.13-4.15). The first set of PDX401 and PDX574 experiments were performed using IP ISRIB and IP paclitaxel. In the PDX401 model, ISRIB appreciably restricted tumour growth relative to both controls and paclitaxel. ISRIB also independently decreased tumour sphere formation and when used in combination with paclitaxel caused the greatest decrease in sphere formation of any treatment in any of our models (Fig. 4.13 and Fig. 4.14). In the PDX574 tumours, there were no statistically significant alterations in tumour size, but there was an increase in the survival of the cohort receiving the combination treatment (Fig 4.15 a). A small survival increase was observed in the mice receiving ISRIB as a monotherapy, but a much larger cohort would be needed to validate this observation (Fig 4.15b). The first set of PDX401 and PDX574 experiments were performed using gavage ISRIB and IV paclitaxel. Under these treatment conditions, PDX574 continued to be resistant to all treatments with no significant changes relative to the control group (Fig 4.16b). In the PDX401 tumours, oral ISRIB did not affect tumour size, but paclitaxel and the combination therapy did, though there was no difference between these two groups (Fig 4.16 a). As there was no toxicity observed in the ISRIB group, ISRIB could have feasibly been continued after treatment to determine if there would have been an effect on recurrence. Consistently throughout our experiments, we have observed a small increase in survivability in mice treated with ISRIB as a monotherapy, and this includes oral dosing (Fig 4.17). ISRIB in both models prevents the acquisition of a CSC phenotype induced by chemotherapeutic stress (Fig. 4.18). ISRIB on its own decreases sphere
formation in the PDX401 tumours relative to untreated controls, though this same effect was not observed in PDX574. ATF4 IHC staining was used to assess alterations in the activity of the ISR. ISRIB decreased ATF4 expression in PDX401 tumour tissues and partially reversed the induction caused by paclitaxel (Fig. 4.19). Due to testing multiple models and alternate means of drug administration the power of individual experiments was often small, however across all experiments ISRIB IP as a monotherapy increased median survival by two days (p=0.013) and oral ISRIB increased survival by 4.5 days (p=0.020). These data suggest that there is a real effect of ISRIB on survival and that dose, dosing schedule, and method of administration should be further optimized and tested on larger cohorts. Our experimental data is well supported by the breast cancer patient data found in the TCGA in which PERK is more highly expressed in tumours than normal breast tissue, and lower levels of PERK are associated with enhanced survival. Finding the means to target this pathway optimally may prove to be valuable in the simultaneous targeting of multiple tumour phenotypes.

There are a few important considerations when interpreting the in vivo experimental design. Mice were always implanted with bilateral tumours. This design was adopted to minimize the number of animals that were sacrificed to assess tumour size and sphere-forming capacity. Mice were sacrificed when either tumour reached 10mm in diameter. The consequences of this are that the chance of a mouse reaching the trait endpoint and being sacrificed are doubled. A single tumour per mouse design would be more sensitive to detecting enhanced survival in a subset of the population. The two tumours per animal model also potentially added some variability to the endpoint of the study. Because tumours were removed in pairs, the sphere formation capacity was not always tested at a tumour diameter of 10mm, in some cases, one tumour was smaller at the time of sacrifice. We observed no evidence that this had an appreciable impact on the final results, but it is worth noting a source of error that can result from the study design. Using tumour sizes as the endpoint for these experiments allowed the recovery of the full diversity of tumour microenvironments including the reestablishment of hypoxic and nutrient gradients. The span of time between final treatment and sacrifice means that some results like those from the PDX401 paclitaxel IV experiment (Fig. 4.16 a) were maintained long after the treatment was withdrawn and thus may be appropriately viewed
as having implications for recurrence or altering the effectiveness of second-line therapies. Insights in the literature and our preliminary results suggest that ISRIB could alter chemoresistance, but further investigation is beyond the scope of this project [597].

Other research questions remain such as, what are the stress and plasticity characteristics of the tumours throughout the course of treatment? A more precise spatiotemporal understanding of plasticity and stress will help identify what predetermining factors allow some cells to adapt when others die, while also resolving the relative importance of plasticity and selection. The most prominent result of PERK inhibitors as cancer treatments was a reduction in metastasis [590]. When this data is taken together with our results that demonstrate that stress signalling through eIF2α can increase colonization in the lung (Fig. 4.11), it becomes clear that exploring the use of ISRIB, or future ISR inhibitors, to prevent metastasis may be an avenue to achieve therapeutic benefit. This would likely take the form a preclinical model in which ISRIB is use as a neoadjuvant therapy prior to tumour resection or chemotherapy.

In this project, we have demonstrated that the ISR is an integral part of the functional acquisition of plasticity in breast cancer. This observation was true across three models representing diverse breast cancer tumour types. Furthermore, we have shown that the mitigation of stress response signalling through the ISR can alter the microenvironmental distribution, CSC frequency and stress responses, though due to a combination of a mouse-minimizing study design, small sample size, and small effect size, we were unable to determine how consistent the effect of ISR inhibition is on cancer progression. Because translation is a central node for not just plasticity and stress responses but part of tumour development, chemoresistance, and energy homeostasis, it is an attractive means of targeting multiple important protumourigenic phenotypes to enhance the treatment options available to patients.
4.3 Methods

4.3.1 Cell Culture and Treatments

T47D cells, obtained from ATCC (Manassas, Virginia, USA), were maintained in RPMI-1640 Medium (Life Technologies; Carlsbad, California, USA) with 10% fetal bovine serum (FBS) (Life Technologies) at 37°C with 5% CO₂. Cells were passaged using 0.25% (w/v) trypsin (Life Technologies) as per ATCC recommendations. MCF7 cells, obtained from ATCC, were maintained in RPMI-1640 Medium with 10% FBS at 37°C with 5% CO₂. Cells were passaged using 0.25% (w/v) trypsin as per ATCC recommendations. SUM149 cells, purchased from BioreclamationIVT, were grown in Ham's F-12 medium with 5% heat-inactivated FBS, 10mM HEPES, 1µg/mL hydrocortisone, and 5µg/mL insulin. All cells were grown in a humidified environment at 37°C with 5% CO₂.

H9 hESCs from WiCell (Madison, Wisconsin, USA) grown on irradiated CF-1 Mouse Embryonic Fibroblasts (GlobalStem; Gaithersburg, Maryland, USA) in knockout DMEM/F12 (Life Technologies; Carlsbad, California, USA), 20% knockout serum replacement (Life Technologies), 1X non-essential amino acids (Life Technologies), 2mM glutamine (Life Technologies), 0.1mM 2-mercaptoethanol (BME; Thermo Fisher Scientific; Waltham, Massachusetts, USA), and 4ng/mL of basic fibroblast growth factor (FGF) (Life Technologies). For experiments cells were passaged into feeder-free conditions. Feeder free conditions consisted of Geltrex matrix (Life Technologies) as a growth substrate and mTeSR1 media (Stem Cell Technologies; Vancouver, British Columbia, Canada).

We employed recombinant human NODAL (R&D Systems, Minneapolis, Minnesota, United States) and SB431542 (10µM) (Sigma-Aldrich) to activated and inhibit NODAL signalling as well as INK128 (MLN0128, 20nM) (Sellekchem Houston, TX USA).

4.3.2 Hypoxia Treatments

Hypoxia was administered at the noted concentrations using Xvivo system (BioSpherix; Parish, New York, USA). The Xvivo’s continuous monitoring was used to ensure consistent and accurate oxygen levels were maintained. Though different oxygen
concentrations were used for different experiments, the temperature and CO₂ were consistent at 37°C with 5% respectively. Upon the completion of the hypoxia treatment, cells were removed and processed for downstream application.

### 4.3.3 Real Time PCR Analysis of Gene Expression

Real time PCR analysis was performed on 1µL of cDNA using TaqMan Gene Expression Master Mix according to the manufacturer’s procedures. FAM labelled TaqMan® gene expression human primer/probe sets (Thermo Scientific). mRNA expression was compared to untreated control using the ΔCT method. Data was collected on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad; Hercules, California, USA) using standard real time PCR settings.

1) Activation 95°C 10 min
2) Melting 95°C 15 sec
3) Annealing/ extension 55°C 1 min. Return to step 2 for 40 total cycles

Melt curve analysis was performed to ensure the production of a single amplicon.

### 4.3.4 Sphere Formation

Sphere formation media was composed of DMEM/F12 + GlutaMax (Life Technologies), 1x B27 (Life Technologies), 20 ng/mL epidermal growth factor (EGF) (Life Technologies), and 10 ng/mL FGF (Life Technologies). After treatment, cells were harvested using 0.25% (w/v) trypsin (Life Technologies), the trypsin was neutralized, and the cells resuspended in fresh media. These cells were filtered through a 40µm pore filter (Thermo Fisher) to obtain a single cell solution. Cells were counted using trypan blue (Thermo Fisher) and diluted in the sphere formation media to the appropriate concentration for plating. Seed 200µL of the diluted cells into each well of a 96 well Ultra-Low Attachment Surface plates (Corning, NY, USA). Spheres were given between 10 and 21 days to grow.
4.3.5 Imaging Spheres

Images of spheres were captured using the EVOS FL Cell Imaging System (Thermo Fisher) at 4X magnification. Minimal changes were made to contrast and brightness to improve the visibility of the spheres.

4.3.6 RNA Extraction

The PerfectPure RNA Cultured Cell Kit (5-Prime; Hilden, Germany) was used to extract total RNA from cultured cells following the manufacture’s protocol using the following optional methods. Lysis buffer was added directly to the plate (option c for cell lysis manufacturer’s instructions). Optional DNase treatment was performed, and RNA was eluted in 50µL. Three (3) µL of purified RNA was used for quantification using the Epoch plate reader (Biotek; Winooski, Vermont, USA).

4.3.7 Reverse Transcriptase PCR and Complementary DNA Synthesis.

cDNA was made from purified total RNA using high capacity cDNA reverse transcription kit (Applied Biosystems; Foster City, California, USA) as per manufacturer’s protocol. The included random hexamers were used to prime reverse transcription and 1µg of RNA was used for each. ‘No Template,’ reactions without RNA and ‘No RT,’ a reaction without reverse transcriptase enzyme were included as controls.

4.3.8 Western Blots

Cells were lysed on-plate using Mammalian Protein Extraction Reagent (M-PER; Thermo Scientific), with Halt Protease Inhibitor Cocktail (Thermo Scientific), and Phosphatase Inhibitor (Thermo Scientific). Protein was quantified according to manufacturer’s instructions utilizing Pierce BCA Protein Assay Kit (Thermo Fisher) and measured on a FLUOstar Omega plate reader (BMG LABTECH; Offenburg, Germany). 4x Laemmli buffer (Bio-Rad; Hercules, California, USA) with 5% BME (Sigma-Aldrich; St. Louis, Missouri, USA) containing 20µg of protein was boiled for 10 minutes and loaded to be analyzed. Samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE), and then transferred onto Immobilon-FL membranes (Millipore). Membranes were blocked with 5% milk in PBS 0.1% Tween (Sigma-
Aldrich) for 1h at room temperature, incubated with primary antibody overnight at 4°C (Supplemental Table 2). After the membranes were washed in PBS 0.1% Tween (Sigma-Aldrich) horseradish peroxidase (HRP)-conjugated secondary antibodies (Bio-Rad) were applied. The membranes were incubated for 1h at room temperature, washed to remove excess secondary antibody, and Clarity Western ECL Substrate (Bio-Rad) was used to detect signal. ChemiDoc™ XRS+ System (Bio-Rad) or film were used to image the western blots. Densitometry was performed using ChemiDoc™ XRS+ System (Bio-Rad).

Florescence western blot detection: Using the Trans Blot Turbo (settings of 25 V and 1.3 A for 15 minutes; Bio-rad) proteins were transferred to a low-auto-fluorescence PVDF membrane (Bio-rad), blocked for one hour at room temperature with Odyssey Blocking Buffer (Li-Cor; Lincoln, Nebraska, USA), then incubated with primary antibody overnight at 4°C in Odyssey Blocking Buffer with 0.1% Tween-20 (Sigma-Aldrich). Membranes were then probed with corresponding Li-Cor anti-mouse or anti-rabbit fluorescent secondary antibodies for one hour at room temperature at dilutions of 1/10 000 in Odyssey Blocking Buffer with 0.1% Tween-20 (Sigma-Aldrich) and 0.1% Tween. Imaging was conducted using the Li-Cor Odyssey Clx imaging system. Scans were performed at intensities that did not result in any saturated pixels at each time point.

4.3.9 In vivo Tumour Implantation

All experiments involving animals were approved by the Animal Use Subcommittee at the University of Alberta (AUP00001288 and AUP00001685).

Orthotopic Xenografts

A total of 500,000 SUM 149 cells in 100 µL RPMI:Matrigel (1:1) were injected into the right thoracic mammary fat pad of 7-8 week old female NSG mice. Mice were randomized, and treatments were administered when tumors reached a maximum diameter of 5 mm. At this point, mice were treated with DMSO vehicle control, ISRIB (2.5 mg/kg IP) or paclitaxel (20 mg/kg IP or 15 mg/kg IV) for the times indicated. Tumour measurements were taken twice per week and a digital caliper was used to measure Length x Width x Depth of the tumour upon excision in order to calculate
volume. Mice were sacrificed when tumors reached \( \sim 1 \) cm in diameter. Tumours were cut in half. One half was of the tumour was dissociated and the other was fixed with 4% formaldehyde, paraffin embedded, sectioned and stained with H&E or used for immunohistochemistry. Survival curves for overall survival were constructed using the Kaplan-Meier method and significance determined by log-rank test.

**Patient Derived Xenografts**

Two PDX models obtained through a collaboration with Oncotest (Charles River, Freiburg, Germany) were used: PDX 401 is a well differentiated basal-like TNBC and PDX 574 is a poorly differentiated basal-like TNBC. Viable pieces (~1mm in diameter) were placed, through a small incision, into the mammary fat pads of 7-8 week-old female NSG mice. At this point, mice were treated with DMSO vehicle controlISRIB (2.5 mg/kg IP or 10 mg/kg by gavage) or paclitaxel (20 mg/kg IP or 15 mg/kg IV) for the times indicated. Tumour measurements were taken twice per week and a digital caliper was used to measure length. Mice were sacrificed when tumours reached \( \sim 1 \) cm in diameter. One half of the tumour was dissociated and the other was fixed with 4% formaldehyde, paraffin embedded, sectioned and stained with H&E or used for immunohistochemistry. Survival curves for overall survival were constructed using the Kaplan-Meier method and significance determined by log-rank test.

**Tail-Vein Lung Metastasis Assay**

SUM 149 cells were pre-incubated as described, then trypsinized and counted. 500,000 cells in 700 uL Ca2+-free HBSS were injected into the tail vein of female NOD-scid IL2Rgamma\(^{null}\) (NSG) mice. Mice were sacrificed at 8 weeks (to tumour formation). Lungs were formalin-fixed and paraffin-embedded, and immunohistochemical staining on this tissue was conducted using a human-specific HLA antibody (Supplementary Table 2) as per the manufacturer’s instructions. For each mouse organ, 3-6 sections were acquired from evenly spaced areas throughout the tissue, and the average number of metastases per mouse organ was calculated.
4.3.10 Tumour Dissociation

Half of the 10mm tumours were fixed and paraffin imbedded for Immunohistochemistry. The other half of the tumour was dissociated using the MACS Miltenyi Biotec Human Tumour Dissociation Kit (MACS Miltenyi Biotec; Bergisch Gladbach, Germany) according to the manufacturer’s instructions prior to enumeration of live cells using trypan blue.

4.3.11 Immunohistochemical Staining

Staining was preformed using the Envison+System HRP anti-mouse IgG (Dako; Santa Clara, California, USA) Formalin-fixed, paraffin-embedded tissue underwent deparaffinization in xylenes, hydration through an ethanol series, antigen retrieval with citrate buffer (Dako), and peroxidase and serum-free protein blocking (Dako). NODAL, HLA, CA9, p4E-BP1, 4E-BP1 or ATF4 specific antibodies (Supplementary Table 2) were applied. Slides were rinsed in TBS-T and treated with Envison+ HRP anti-mouse IgG (Dako). Color was produced with DAB (brown) substrate (Dako) and counterstained with Mayer’s haematoxylin (Vector Laboratories, Burlingame, California, USA). Samples were dehydrated in reagent grade alcohol and cover slipped with permanent mounting medium. Negative control reactions were conducted with mouse IgG, isotype controls used at the same concentration as the primary antibodies.

4.3.12 Analysis of Patient Data

Level 3 TCGA RNAseqV2 BRCA gene expression data and clinical information was obtained from the TCGA Data Portal in April 2015. RNA-sequencing RSEM values were used in downstream analyses. For TCGA RNA-seq samples, relative abundance (transcripts per million, TPM) was calculated by multiplying the scaled estimate data by 106 and used in downstream analysis. We conducted all analyses and visualizations in the RStudio programming environment (v0.98.501). R/Bioconductor packages ggplot2, survminer, pROC, and survival were used where appropriate. Patients were dichotomized by median expression PERK into two equal groups Low EIF2AK3 and High EIF2AK3. Student’s t-test was used to evaluate expression differences between cohorts. The associations between PERK, and overall survival were tested in univariate Cox regression
models with considered as continuous variables (as log [RSEM expression values+1] or binarized by ROC curves). Survival curves for overall survival were constructed using the Kaplan-Meier method and significance determined by log-rank test.
References


Chapter 5

5 General Discussion

In this study we have identified selective translation as a central node through which the gene expression required for EMT, plasticity, and stress response expression are coordinated. Furthermore, we have identified many previously undescribed 5′UTRs belonging to plasticity and EMT regulating mRNAs which display differential translation efficiency in response to stress. The induction of plasticity through selective translation enhances tumourigenicity by increasing CSCs and metastasis. By inhibiting pluripotency factors like NODAL, or by limiting PERK/eIF2α signalling, plastic adaptation to stress can be prevented. We demonstrate that these types of stresses are an intrinsic aspect of the tumour microenvironment that are further amplified by chemotherapy. These data help fill an important mechanistic gap in our understanding of how niche microenvironmental stresses drive the acquisition of tumourigenic and CSC phenotypes. Throughout an individual tumour’s natural history, cells within the tumour will likely experience this process repeatedly, leading to diverse phenotypes and progressive dedifferentiation. We are only now beginning to understand the breadth and sequence of these types of microenvironmental effects.

The heterogeneity of breast cancer has been observed for decades [12, 14]. For just as long we have recognized the loss of cellular identity as a key feature of malignancy [14, 17]. Built upon these observations, cancer biologists from myriad scientific disciplines have discovered many of the mechanisms that drive these changes. New techniques like deep sequencing have expanded our understanding of the development of cancer, as well as the effects that treatments have on altering tumour heterogeneity. These experiments support the concept that the accumulation of mutations is a continuous process that is so prevalent that in some tumours virtually every cell contains unique mutations [65]. As previously discussed, this level of mutation leads to two categories of heterogeneity; intertumoural and intratumoural [65]. Intratumoural heterogeneity can be conceptually subdivided into spatial and temporal heterogeneity. Tools like deep sequencing, single-cell sequencing, and laser capture microdissection can be used to analyze tumour cells from different
Throughout the process of transformation, a series of stress events each elicit a survival response. This includes an upregulation of processes that maintain homoeostasis (glycolysis, stress responses) while also increasing the expression of factors associated with EMT—SNAIL, SLUG, TWIST, VIM, and ZEB. By altering translation, limited cellular resources become restricted towards cell survival and plasticity including stem cell factors like NODAL, NANOG, OCT4, and SOX2 as well as factors that alter cell fate and plasticity. This includes an increased expression of glycolysis enzymes, an increased expression of EMT factors, and a decreased expression of translation initiation factors. This process, driven by factors like pro-tumorigenic phenotype factors and chemoresistance, results in the survival of cancer cells, thereby driving tissue dedifferentiation.

Figure 5.1: A Model of How Hypoxia and Stress Promotes Pro-Tumorigenic Phenotypes in Cancer.
areas within a tumour or from different metastatic sites to determine the extent of phenotypic and mutational heterogeneity. These types of differences are what define spatial heterogeneity. Stress, treatments, or the acquisition of new mutations change the molecular characteristics of cancer cells over time [598-601]. How a tumour changes and develops can be termed temporal heterogeneity. Barcoding and lineage tracing are powerful tools for understanding how diverse phenotypes are acquired [602]. Both endogenous and exogenous forces drive the acquisition of heterogeneity [603, 604]. Though we now have the tools to investigate the intricacies associated with heterogeneity, we still possess an insufficient understanding of the complexity required to address the two wide-ranging drawbacks of current therapies. The first, that treatments that have broad effects like radiation and chemotherapy have extensive side effects, and second, that highly targeted treatments are likely to show limited effectiveness due to clonal and phenotypic heterogeneity.

5.1 Reexamining and Expanding Evolutionary Models

The foundational cellular alterations upon which a tumour develops are mutations. Hematological tumours and solid tumours both progressively acquire mutation but show different patterns of acquisition. Solid tumours have a complex branching pattern of mutational evolution leading to a more diverse genetic makeup than the more linear profiles found in hematological cancers like acute myeloid leukemia [605, 606]. The most obvious consequence of population diversity in stressful environments is the selection and propagation of the most aggressive clones, but new observations suggest a much more nuanced system. If there were even a small selective advantage, a simple evolutionary model would suggest the emergence of a dominant clone. In certain scenarios in which cells from a solid tumour experience a strong selective pressure this phenomenon can be observed [598-600]. The distribution and rate of events that create genomic instability are the consequence of cell intrinsic and extrinsic factors. Genomic instability exists in a reciprocal relationship with the tumour microenvironment. Mutations provide a selective advantage, but these cells require a blood supply, nutrients, and oxygen to thrive. If these factors are in insufficient supply, there exists a selective pressure from which further subclones will be enriched. The geographic isolation of
subsequent branching subclones produces the genomic and phenotypic heterogeneity that is observed in solid tumours. Microenvironmental factors like hypoxia then exert their effects increasing genomic instability [603]. This theory extrapolates upon data from barcoding and lineage tracing experiments, as well as data indicating that microenvironmental stress can increase genomic instability and hypothesizes that repeated spatially distinct cycles support the acquisition of genetic and phenotypic heterogeneity [598-600, 603]. Verduzco et al. provide experimental evidence for the role of cyclical microenvironmental insults — hypoxia — in selecting protumourigenic genotypes and phenotypes [601]. By testing both intermediate and chronic hypoxia, the authors determined that 50 cycles of intermediate hypoxia generates heritable decreases in p53 and E-cadherin and an increase in HIF-1α detectable even after the cells were returned to normoxia for two months. Through this process, MCF10A cells developed enhanced survival, invasion, and therapy resistance. When these data are taken together with earlier work showing that rare mutants with a selective advantage are enriched by exposure to hypoxia we create the framework for the progressive and reciprocal interaction of genetic instability and the tumour microenvironment in cancer development [215].

Diversity between populations of cells is not solely determined by the concept of Darwinian selection because this model cannot account for the observed stable phenotypic heterogeneity within tumours, even among cells with the same genetic background. Though it is difficult to address these problems, through a combination of modern techniques like DNA barcoding, single cell dilutions, and single cell sequencing, they can be addressed. Phenotypic heterogeneity among clones was tested by barcoding breast cancer cells, identifying them as having an epithelial or mesenchymal phenotype, and then expanding the cells from single clones for up to one month [602]. Despite the colonies being derived from a single cell, the majority of colonies contained progeny of both phenotypes [602]. Furthermore, the authors showed that the ratio of epithelial to mesenchymal cells of each clone was maintained between the set of genetically identical cells in the parent population and colonies derived from single cells to produce a new clonal population [602]. These data provide strong evidence that phenotypic plasticity is an inherent and stably inherited state. Like other phenotypes, phenotypic plasticity can
produce a fitness advantage. The application of therapies in this study selected for cells that were more capable of switching phenotypes. The author's work showed promising evidence that targeting both states may be a method to more effectively target adaptable cancer cells [602]. We know, however, that other critical phenotypes can be altered to evade therapy, so if switching states or phenotypes is the important aspect of adaptation, an alternate strategy may be to target the mechanism that allows switching to occur. Under conditions like hypoxia or chemotherapy where translation mediates a transition between cell states concomitant with other cellular stress responses, targeting this process may be a means of addressing multiple concurrent adaptive phenotypes.

If there exists phenotypic heterogeneity within single clones producing different cell states — epithelial and mesenchymal — it is reasonable to hypothesize that this is true for other phenotypes. Because this trait is encoded and maintained across generations and multiple clones are maintained within a tumour there is a rationale to hypothesize further that heterogeneity may provide a selective advantage. This concept is called interclonal cooperation. In a series of experiments by Cleary et al. they identified coexisting mutational sub-clones, basal HRas\textsuperscript{mut}/Wnt1\textsuperscript{low} and luminal HRas\textsuperscript{wt}/Wnt1\textsuperscript{high}, whose propagation and relapse after targeted therapy requires Wnt from high Wnt1 expressing cells [607]. This cooperation demonstrated integrated expression between two clones as one example in which cells could alter their microenvironment through the maintenance of heterogeneity.

It is important to have a theoretical framework for how clones are maintained between the initial instance of mutagenesis from which a new clone arises and the next mutational event. Geographic isolation resulting in growth in different microenvironments and interclonal cooperation provide two different mechanistic explanations for how heterogeneity can be maintained which complement and advance the metaphor of Darwinian evolution that is commonly used to describe clonal selection. The two evolutionary phenomena that are analogues for these tumourigenic processes are speciation through geographic isolation and symbiosis.
5.2 Heterogeneity, Stress, and Phenotypic Plasticity

Though it is tautological to say that cells within a tumour have diverse responses to treatments and that those cells that respond least develop into resistant disease resulting in recurrence, this framing is still valuable in understanding the problems that arise from heterogeneity. A broad range of cellular phenotypes results from the combination of genetically diverse cells existing in a multitude of microenvironments which in turn alter gene expression in unique ways. Current breast cancer therapies affect subpopulations of cells differently, their efficacy often dependent on the presence of a specific phenotype, like high levels of mitosis. Paclitaxel resistance can occur when proliferation rates and mitotic entry cease as its primary means of cytotoxicity requires mitosis [608-610]. Likewise, cancer cells can enter a senescence-like-state in response to genotoxic stress, radiotherapy, and chemotherapy [611, 612]. Senescence is often viewed as an indication of treatment response because it is a cytostatic phenotype that can lead to apoptosis, but Achuthan et al. demonstrate a process by which cancer cells differentially respond to treatments leading to chemoresistance [604]. Apoptosis is induced in the majority of the MCF7 cancer cells when chemotherapeutic agents are applied. However, these treatments resulted in selective enrichment of a subset of cells that entered drug-induced senescence, a state which enriched for CSC properties [604]. These resistant cells possessed high levels of ROS, but occasionally asymmetrically divided giving rise to cells expressing lower levels of ROS and CSC phenotypes that were even more resistant to therapy than their parental line. This result means that these two different cell states require initial selection followed by the secondary induction of a low ROS stem-like state [604]. The resistance observed was shown to be Nrf2/OCT4 dependent [604]. These experiments are a detailed example of a process by which the initial heterogeneity in cancer lines provides protection against cancer therapy but, through both selection and the induction of a stem-like state, that ultimately gives rise to phenotypic heterogeneity and resistance thereby promoting disease development. This process of selection and adaptation represents a single instance among thousands that diversify and select cells in any single tumour and provides an example by which to understand how stresses like hypoxia can affect disease phenotypes.
5.3 Hypoxia Causes Cells to Adopt a CSC State.

Hypoxia has been identified as a source of genomic instability, clonal selection, translation inhibition, plasticity, and heterogeneity, which collectively create the conditions for tumourigenesis and resistance [236, 613-615]. In both MCF7 and MDA-MB-231 breast cancer cell lines, cells exposed to an *in vitro* tumour microenvironment, as identified by an HRE-EGFP expression system, increased the frequency of CSCs along with a host of tumorigenic phenotypes including clonogenicity, invasion, migration, and tumour growth [236]. What was most interesting was that these traits were stably maintained for multiple generations of *ex vivo* cell culture in normoxia [236]. In the aggressive murine breast cancer cell line 4T1, breast cancer stem cells were demonstrated to be dependent on CA9 expression. Knockdown of CA9 decreased EMT and pluripotency [253]. These decreases in stemness also increased the cell’s sensitivity to chemotherapy *in vivo* [253]. There is now evidence that this may limit the effectiveness of antiangiogenic therapies [470]. Tumours from mice treated with antiangiogenic therapies have higher levels of hypoxia. Within these areas, the frequency of CSCs was increased [470].

The interactions between hypoxia and CSCs are multifaceted. Hypoxic microenvironments shield cells from the effects of treatments while also promoting plasticity which produces a set of coincident treatment-resistant phenotypes that serve to protect the cells most capable of repopulating the tumour, leading to recurrence [284, 604]. An important aspect to many of these experiments is that the changes induced by hypoxia are heritable, and in many of our experiments there was no evidence to support large-scale cell death and clonal selection, which supports the hypothesis that hypoxia induces the plastic switching of cell state which may result from epigenetic alterations [236]. To that end, we demonstrate an interconversion of nearly 50% of SUM149 cells from non-CSCs to a CD24<sup>low</sup>/CD44<sup>high</sup> CSC phenotype with a 24h hypoxia treatment. To acquire this level of conversion from selection alone, it would require cell death of approximately 97% of the non-stem cell population to achieve the one-to-one CSC to non-CSC ratio observed (Fig. 2.4b). In these experiments, we observed no significant difference in cell death that would have been sufficient to explain the results. This result
represents a significant change in cell identity and supports the theory that plasticity is mutable and can be acquired in response to stress. To confirm these results, cells from two different breast cancer cell lines — T47D and MCF7 — were exposed to hypoxia which resulted in an increase in their capacity to form spheres.

To elucidate potential drivers of adaptive plasticity, we inhibited Nodal signalling in cells exposed to hypoxia, which reversed the induction of sphere formation indicating that Nodal is an integral part of the signalling network required for hypoxia-induced pluripotency. Based on our RNAseq and gene expression data, Nodal is one of many potential drivers of plasticity that are regulated by hypoxia (Fig. 2.2, Fig. 2.3, Fig. 2.4). We corroborated these findings in several ways. First, we did so by using rhNodal to induce both sphere formation and the CD24<sub>low</sub>/CD44<sub>high</sub> CSC phenotype in breast cancer cell lines (Fig. 3.7, Fig. 2.4a). Second, the stably selected Nodal overexpression SUM149 cell line showed an increase in the stable levels of CD24<sub>low</sub>/CD44<sub>high</sub> cells (Fig. 2.5). We know from our staining of the PDX tumours that Nodal expression is located in hypoxic regions generally correlating with CA9 (Fig. 3.18). We also determine that CA9 is expressed in areas of active 4E-BP1 indicating translational repression in these regions (Fig. 3.15). Finally, we show that in three different tumour models, cells expressing high levels of Nodal also expressed high levels of CA9 (Fig. 2.6). Our cell line models respond to both an increase in endogenously expressed Nodal as well as the application of exogenous Nodal. Taken together with the feed-forward loop created by Nodal expression in response to hypoxia which we have described previously, it suggests that Nodal can act in both a paracrine and autocrine manner to induce and sustain plasticity [135]. Because Nodal has been demonstrated to regulate epigenetic modifications via the recruitment of Jmjd3, it is a sound hypothesis that Nodal expression is part of the mechanism responsible for the hypoxia-induced heritable plasticity observed by other groups [236, 253, 616]. Throughout our experiments, we demonstrate a broad co-regulation of factors that control cell fate and stress pathways that are activated under hypoxic conditions. The expression of factors regulating EMT, plasticity, angiogenesis, and stress response, are all simultaneously upregulated by stress in breast cancer (Fig. 2.2, Fig. 2.3, Fig. 2.4, Fig 3.2, Fig 4.5c). In order to achieve expression against a background of robust translational repression, a subset of mRNAs necessary for survival
must circumvent this repression. Broadly, we observe that the stress-induced activation of pathways that decrease translation result in the upregulation of markers of plasticity and EMT and drive protumourigenic phenotypes like metastasis, chemoresistance, anchorage-independent growth, sphere formation, and CSC abundance.

5.4 Translation and Non-Coding mRNA sequences Facilitate the Acquisition of Plasticity in Response to Hypoxia

In a paper by Akrap et al. the authors used single-cell PCR to delineate the sequence of events occurring as cells dedifferentiate in response to hypoxia. Using PCR to detect a small set of only 22 genes representing five different phenotypes, differentiation, proliferation, EMT, breast CSCs, and pluripotency, they were able to determine that hypoxia and 3D cell culture induced changes in the proportion of cells expressing plastic phenotypes [617]. Hypoxia enriched for two distinct clusters of stem cells. The first showed high expression of plasticity and EMT markers — NANOG, SNAI1, SNAI2, and FOSL1 — and the second showed the highest levels of CSC markers — ABCG2, ALDH1A3, and CD44. Both sets of cells also had lower levels of proliferation than cells found in normoxia [617]. From their single-cell data, the authors of this study used principal component analysis and self-organizing maps to demonstrate that hypoxia and 3D culture enriched the stem cell-like fractions of the described hierarchically organized phenotypic states. These states are defined by the transition from differentiated cancer cells through to a quiescent CSC state; the transition of which is characterized by the sequential acquisition of EMT gene expression, pluripotency markers and low levels of proliferation [617]. Whereas this study does an excellent job of demonstrating the plasticity of cell state and heterogeneity found within a single cancer, it was beyond the scope of the project to determine the mechanism by which this transition occurs. We show complementary results using both IHC and flow cytometry to identify that the subset of breast cancer cells expressing high levels of the hypoxia marker CA9 also expresses the highest levels of the pluripotency factor Nodal (Fig. 2.6, 3.18).

Hypoxia comprises a multitude of signalling responses that govern many phenotypes. Because we witnessed extensive translational inhibition, approximately 80%, as well as
discordance between Nodal transcription and protein expression, we posited that translation might be a hub from which multiple pro-survival phenotypes are activated in response to stress (Fig. 2.8, Fig 2.9). First, we identified a number of transcripts representing multiple pro-survival phenotypes that are translationally maintained in hypoxia. The phenotypes represented by our genes of interest are survival (ATF4), angiogenesis (VEGF), and glycolysis (GAPDH), along with several EMT and stem cell markers. We observe that most EMT and stem cell factors had multiple detectable 5′UTRs, the notable exception being SLUG. By using real-time RT-PCR we confirmed that breast cancer cells simultaneously produce multiple mRNAs that possess different 5′UTRs yet encode the same protein product. Then we subsequently confirmed that these mRNAs are translationally active, as determined by ribosome occupancy. The mRNA isoforms of genes regulating plasticity with different 5′UTRs exhibit different translational efficiencies in both standard growth conditions and in response to hypoxia. Because the field is still in the early discovery phase, it is difficult to say which sequences within the 5′UTR are responsible for changing translational efficiency but we did discover one uORF in the NODAL 298 5′UTR. We mutated this AUG to an AUA which decreased the efficiency of the basal level of translation and prevented the upregulation in response to hypoxia (Fig. 2.19b). These data suggest that large-scale translational reprogramming is occurring during stress to mount a multi-phenotype pro-survival response. Polysome RNAseq experiments could provide valuable insights into the full extent of this response while producing the data necessary to look for important sequence similarities in the 5′UTR and 3′UTR of these mRNAs. Repeating these experiments with chemotherapy or radiation-induced stress with and without perturbation to the translational response would provide a valuable mechanistic understanding of how treatment and translation contribute to tumourigenesis and treatment resistance.

Here we establish a mechanistic connection between hypoxia, stress response pathways, and plasticity by demonstrating that the same mechanism, translation, responsible for redressing homeostasis also enriches plasticity. Though attempts have been made to elucidate the mechanisms by which this occurs such as sequence length, secondary structure, and sequence elements like uORF, 5′ terminal oligopyrimidine tract (5′ TOP), and GC rich (GGC)-repeat motifs, the interactions all of the constituent-parts by which
any one mRNA is regulated is still unclear [375-380]. When considering the expression of families of genes or co-regulated networks of genes, especially transcripts that are expressed during times of stress, it is important to consider the conservation of non-coding sequences that may alter expression post-transcriptionally. It is likely that through the pursuit of a greater understanding of translation and stress we will discover important translational facets integral to gene expression during less dramatic cellular responses, for which development is a clear candidate.

5.5 Phenotypic Plasticity is Enhanced in Response to mTOR/4E-BP1 Signalling.

We establish that the overexpression of 4E-BP1, like that observed in breast cancer relative to normal tissue, increased the plasticity of cancer cells in vitro concomitant with an increase in Nodal expression (Fig. 3.4, Fig. 3.6). Direct inhibition of mTOR also increased plasticity and CSC frequency in a Nodal-dependent manner, but the entirety of the phenotypic enhancement is likely owed to broader increases in EMT and plasticity gene expression (Fig 3.5, Fig. 3.7, Fig. 3.2). We also show that the inhibition of mTOR results in the inactivation of eIF2α, which likely occurs through eIF2B (Fig. 3.1) [514]. The connection between these signalling pathways has been observed in studies of autophagy in which the interplay between mTOR and PERK signalling is responsible for mediating the decision to mount a survival or apoptotic response [618, 619]. Understanding this type of crosstalk is important because the unwanted activation of pathways downstream of mTOR resulting in the diminished efficacy of mTOR inhibitors has been a problem in previous generations of this class of drugs [620].

Our in vivo work also demonstrates that mTOR inhibition by INK induces CSCs as measured by sphere formation, and that this effect is mitigated with the coadministration of ISRIB, suggesting that the activation of the ISR is a driver of the plasticity response to mTOR inhibition (Fig 3.11). Because the efficacy of mTOR inhibitors is known to be limited, it is unsurprising then that across our in vivo models INK showed the greatest variability of any treatment, decreasing tumour size in SUM149 tumours but increasing it in the PDX401 line (Fig. 3.9, Fig. 3.16). In the SUM149 and the PDX574 tumours INK and ISRIB+INK decreased tumour size and increased survival. These results mean that in
addition to the cytostatic effects of mTOR inhibition on tumour growth that have been reported in the literature, there is also a simultaneous induction of the countervailing protumourigenic phenotype, plasticity. Our tail vein metastasis assay suggests that stress prior to entering the bloodstream, like hypoxia at the primary site, can prime cells for colonization in the lung (Fig. 3.13, Fig. 4.11). This assay provides a mechanism by which hypoxia drives metastasis and helps explain why metastasizing cells possess increased plasticity [621-623]. It is this balance between competing forces that will determine the effectiveness of therapies designed to inhibit mTOR. To that end, when INK was combined with ISRIB tumour sphere formation decreased and the survival of mice bearing bilateral tumours is increased (Fig. 3.11, Fig. 3.10, Fig 3.19). It is clear that the combination of these therapies shifts the balance of phenotypes toward anti-tumour effects, though it has yet to be determined if this is sufficient to increase the viability of mTOR inhibition clinically.

It is also important to note that mTOR inhibition and eIF2α phosphorylation appear to be aspects of the natural state of ESCs. Further work is necessary to determine to what extent the translational alterations observed in cancer cell lines are an activation and commandeering of a normal and functional system and which aspects are dysregulated through epigenetic or genetic changes [624, 625].

5.6 Addressing the Protumourigenic Effects of mTOR Inhibition

The fact that ISRIB reduces plasticity without altering the efficacy of INK’s inhibition of tumour growth suggests several interesting hypotheses. The first being that limiting eIF4E cap availability and limiting ternary complex availability affect different types of transcripts and ultimately different phenotypes. Our results would suggest that inhibiting translation from eIF4E while preventing the expression of those mRNAs that rely on eIF2α phosphorylation produces the greatest antitumourigenic effects. The second is that the anti-tumorigenic effects of mTOR inhibition are not the result of reduced translation but other downstream effects that result from diminished mTOR activity. This second theory seems like less likely because newer mTOR inhibitors are more effective at
inhibiting 4E-BP1 phosphorylation and have shown greater anticancer effects than their predecessors [544, 626, 627].

Another complicating factor is that central regulators that have broad roles and interact as part of complex signalling networks, like mTOR, may be more susceptible to emergent compensatory mechanisms [544, 627]. A rational approach to deriving useful and translatable therapies from data regarding these high-level pathways would be to investigate their downstream effectors and to identify those responsible for the desired phenotypic changes. In this way, we have tried to limit the indirect activation of the ISR by mTOR inhibitors to narrow the set of affected phenotypes preventing an increase in plasticity with the express purpose of improving these therapies. It may be possible to determine downstream effects more precisely if a unique subset of mRNAs that respond to the different translation inhibitory pathways could be identified. One could measure the translational activity of cancer cells by polysome profiling under conditions of mTOR inhibition in the presence or absence of ISRIB which would allow us to determine which mRNAs are selectively translated by each mechanism. This type of experiment could help to identify mRNA sequences or translation proteins associated with the most detrimental translation circuits, further refining treatment, and even in the absence of targetable entities would deepen our understanding of translation.

Despite the growing understanding of the complexity of these interactions, this may only be the tip of the iceberg. We observed that rhNodal increases the expression of 4E-BP1 (data not shown), likely leading to further translational repression as we see in the 4E-BP1 overexpression experiments (Fig. 3.4). New evidence is also amassing for the role of SMADS in regulating translation; the effect of which has been observed in cancer and in stem cell differentiation [628, 629]. In hESCs hypoxia helps maintain pluripotency, it increases Nodal expression, and it causes the dephosphorylation of 4E-BP1 and the phosphorylation of eIF2α (Fig. 2.11) [135, 188, 630, 631]. Exploring the role of translation in pluripotency and differentiation may provide valuable insights into the potential role stem cell factors play in the coordination of translation.
5.7 ISR in General Stress Tolerance

Both mTOR and the ISR are points of convergence for multiple stresses [488, 549]. Several pieces of data from this study suggest that there is an intrinsic activation of the ISR in our models; hypoxia is present to varying extents in all our models; ATF4 is expressed in all control tumours; in the highly hypoxic PDX574 tumours ISRIB administered as a monotherapy reduces baseline ATF4 expression; and ISRIB as a monotherapy decreases the amount of hypoxia tolerated in the tumour. Furthermore, given further insults like chemotherapy or mTOR inhibition, ISRIB prevents the increase of ATF4 indicating an attenuated stress response (Fig. 3.12, Fig. 4.). The ISRIB-induced mitigation of stress responses decreased the tumour’s capacity to tolerate hypoxia, which manifests as a decrease in the hypoxic area found in tumour sections (Fig. 3.14, Fig. 4.12). ISRIB had the most profound effect on tolerance to tumour hypoxia though it also showed a promising effect in combination with INK and paclitaxel.

5.8 The Role of ISR in Chemotherapy Resistance

We show that paclitaxel induces signalling that restricts translation, resulting in the activation of 4E-BP1 and eIF2α phosphorylation (Fig. 2.12). Treatment of cells with ISRIB sensitizes cells to paclitaxel implicating ISR activation in chemoresistance (Fig 4.6). Translation inhibition profoundly alters gene expression as well as the energy dynamics of cells; thus the precise source of the effects downstream of translation remain unresolved. The mTOR and ISR pathways have been shown to induce quiescence and chemoresistance in multiple cancers including glioblastoma, prostate, colorectal, and pancreatic cancer [632-639]. Because numerous therapies, like paclitaxel, are dependent on rapid cell division, translation inhibition associated cycle arrest may contribute to niche resistance [640-644]. Based on our results and those found in the literature, resistance as produced by restricting translation is likely a multifaceted response that involves decreased cell division, intensified stress responses and elevated plasticity, the result of which enriches chemoprotective phenotypes while favoring the capacity to dynamically adapt cell state to changing microenvironments [434, 604, 645]. There are several plausible non-mutually-exclusive hypotheses to explain how preventing translation mediated adaptations can alter chemosensitivity; preventing cell cycle arrest;
blocking adaptation through translation may prevent the upregulation of proteins like BCL2 and ABC transporters; preventing survival signalling like that of ATF4; or preventing the induction of CSCs (Fig 4.4, Fig. 4.10, Fig. 4.19) [646-648]. There may even be an effect on the non-transformed aspects of the tumour microenvironment. In one study, by decreasing mTOR activity, the microenvironment was altered thereby promoting tumourigenicity by activating the ‘senescence secretory switch’ which induces a chemoprotective secretory response in endothelial cells [649]. As both the ISR and the mTOR pathway are hubs that integrate signals from multiple sources and they regulate a range of proximate phenotypes, manipulating these pathways can have dramatic effects on cellular processes. However, the complexity may obscure the drivers of the ensuing phenotypes or even the phenotypes themselves.

5.9 Translation Mediated Adaptability Limits the Effectiveness of Therapy.

Though there are likely many shared pathways through which these stresses can exert protumourigenic pressures, translation regulation is a nexus for the maintenance of homeostasis and wide-reaching changes in gene expression. Hypoxia, mTOR inhibition, and chemotherapy induce signalling associated with translational reprogramming through the ISR. Translation thus acts as an important phenotypic switch that alters a spectrum of tumourigenic phenotypes including plasticity and stress response.

Having identified Nodal as being translationally regulated, we establish that Nodal’s expression is increased by chemically induced UPR stress (Fig 2.8, Fig. 4.1, Fig. 4.2). This induction occurred along with a network of other plasticity factors (Fig. 4.3). Nodal expression is diminished with the administration of ISRB suggesting that the baseline level of Nodal expression is supported by activation of the ISR via eIF2α phosphorylation. The induction of sphere formation that results from exposure to hypoxia as well as the expression of several EMT markers are dependent on the phosphorylation of eIF2α (Fig. 4.5a, b). In this way, we show the direct role of eIF2α in stress-induced cell fate alterations.
Our data shows the importance of the ISR in hypoxia, exposure to chemotherapy, and in mTOR signalling, but we did not address whether the ISR was relevant to cells in which 4E-BP1 is overexpressed. As the TCGA data seems to suggest that 4E-BP1 is the most commonly overexpressed protein in the mTOR pathway, this model may be the most clinically relevant. The permutations tested are sufficient to conclude that the ISR is an important aspect of multiple stress responses that ultimately increase plasticity and in concordance with the coordination of other stress responses, but are insufficient to determine the temporal nuances or specific mechanisms through which these pathways are regulated.

Hypoxia and chemotherapy potentiate short- and long-term cellular adaptation. We have described many aspects of short-term adaptation including the translational upregulation of mRNA associated with angiogenesis, stress response (ATF4), as well as plasticity and EMT. Among the latter set of genes those regulating plasticity and EMT are several factors that remodel the epigenome [464, 616, 650, 651]. As hypoxia is an early emerging selective pressure in solid tumours, the induction of alterations to the epigenome is an attractive hypothesis to explain the observed stable non-mutation driven alterations in gene expression that advance many tumour phenotypes [455]. Changes in epigenetic regulation and gene expression may be responsible for regulating alterations in the sequences of 5’UTRs. The mechanisms include but are not limited to TSS switching, UTR splicing, intron retention, and translational start site utilization, which all act to alter the included regulatory moieties. Evidence of these mechanisms was observed in our survey of 5’UTRs belonging to EMT and plasticity-related mRNAs, though many were not investigated because other post-transcriptional variations confounded the presence of the 5'UTR. The results of our in vivo experiments suggest several potential benefits to using ISR inhibition as a cancer therapy; use as a monotherapy in resistant disease dosed either orally or by injection; as a means of increasing the efficacy of mTOR inhibitors; coadministration with low does cytotoxic chemotherapy; or potentially as an antimetastatic agent. First, across our models IP ISRIB — 2.5mg/kg every second day — increased median survival by 2 days and oral IP ISRIB — 10mg/kg every day — increased median survival by 3 days (Wilcoxon rank sum, p<0.05). Our studies differ from others in the literature in that we used an active comparator and we tested multiple
modes of action. It is clear from the IHC that our treatments are effectively
downregulating the signalling of demonstrated protumourigenic pathways. Furthermore,
in the case of INK and paclitaxel, across models, inhibition of eIF2α phosphorylation
prevents activation of pro-tumourigenic molecular mechanisms that increase plasticity.
Many of the changes that result from both ISRIB and paclitaxel were detectable weeks
after treatment-withdrawal suggesting that they were heritably maintained which may
have implications on subsequent rounds of treatment in resistant disease. Under certain
circumstances we observed ISRIB improving survival, tumour size, or both. These were
secondary endpoints for our study as our primary goal was to assess plasticity in tumours
in which size was controlled, and treatment termination was standardized between each
arm. We remain optimistic that trials specifically designed to measure maximum
response in tumour size or survival would enhance the current findings.

Based on the observed changes in gene expression during intrinsic tumour stress, and the
phenotypes that ISRIB alters, it is worth considering testing two other treatment courses.
First, ISRIB may be viable as a neoadjuvant therapy. We observed that the reduction of
sphere formation and ATF4 were observable long after treatment withdrawal and that
ISRIB decreased the amount of hypoxia in solid tumours. As cancer stem cells and
hypoxia are associated with treatment resistance, pretreatment with ISRIB may sensitize
cells to therapy. The second option is that, because ISRIB showed no toxicity in our
mice, and CSCs are hypothesized to be the cells responsible for distant relapse, ISRIB
may be a viable adjuvant therapy.

Translation is a precisely regulated process through which high levels of proliferation are
maintained in the bulk population of tumour cells, but data from primary tumours
supports the idea that there is selection and enrichment of tumour cells capable of
extensive translational repression — PERK and 4E-BP1 high. Our studies demonstrate
how translational repression promotes and maintains tumourigenicity, plasticity, and
metastasis, explaining the poor outcomes of patients with higher levels of PERK and 4E-
BP1. Accounting for and mitigating the adaptability of tumour cells by homogenizing the
translational response is a novel means of regulating plasticity while offering an
innovative path to addressing patient’s needs.
References


Supplemental Figure 1: Pooled Polysome Profile Fractions.

Representative polysome profile from MCF7 cells demonstrating the fractions from which monosomes, Low MW polysomes and High MW polysomes were taken.
Supplemental Figure 2: Cage Data From the ENCODE/RIKEN polyA+ CAGE Data Set Showing 5' UTR Utilization with Differentiation.

5'UTRs viewed using UCSC genome browser identifying both annotated SNAIL 85 and the newly discovered SNAIL 417 in H1 HESCs and BJ human foreskin fibroblasts showing that the relative abundance may be altered throughout differentiation.
Supplemental Table 1: Primer Probes Used.

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**Supplemental Table 2: Antibodies Used in This Study**

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<td>HIF1A</td>
<td>10006421</td>
<td>WB</td>
<td>1:1000</td>
<td>Cayman</td>
<td>Anti-HLA Class I ABC antibody [EMR8-5] Mouse mAb Used to detect human MDA-MB-231 breast cancer cells within mouse lungs. Species reactivity: Human</td>
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<tr>
<td>HLA</td>
<td>ab70328</td>
<td>IHC</td>
<td>1:150</td>
<td>Abcam</td>
<td>Anti-HLA Class I ABC antibody [EMR8-5] Mouse mAb Used to detect human MDA-MB-231 breast cancer cells within mouse lungs. Species reactivity: Human</td>
</tr>
<tr>
<td>IRDye 680 Donkey Anti-Mouse</td>
<td>926-68073</td>
<td>Secondary</td>
<td>1:10000</td>
<td>Li-cor</td>
<td>IRDye 680 Donkey Anti-Mouse 926-68073 Secondary 1:10000 Li-cor</td>
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<td>IRDye 680 Donkey Anti-Rabbit</td>
<td>926-68074</td>
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<td>Li-cor</td>
<td>IRDye 680 Donkey Anti-Mouse 926-32212 Secondary 1:10000 Cell Signaling</td>
</tr>
<tr>
<td>Antibody Description</td>
<td>Catalog #</td>
<td>Format</td>
<td>Dilution</td>
<td>Source</td>
<td>Notes</td>
</tr>
<tr>
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<td>--------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
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<tr>
<td>IRDye 800 Donkey Anti-Rabbit</td>
<td>926-32213</td>
<td>Secondary</td>
<td>1:10000</td>
<td>Cell Signaling</td>
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<tr>
<td>Labeled Polymer-HRM-ANTI-Mouse</td>
<td>K4007</td>
<td>IHC</td>
<td>1:1000</td>
<td>Dako</td>
<td>Rabbit mAb. Western blot analysis of extracts from NCCIT, NTERA-2 and iPSCs using Nanog (D73G4) XP® Rabbit mAb. Citations: 71.</td>
</tr>
<tr>
<td>Nanog</td>
<td>4903</td>
<td>WB</td>
<td>1:1000</td>
<td>Cell Signaling</td>
<td>Rabbit mAb. Antibody was validated by pathologist Dr. Martin Koebel, University of Calgary, AB Canada using knockout cell lines and tumour microarrays. Species reactivity: Human. Citations: 11.</td>
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<tr>
<td>Nodal</td>
<td>PA5-28486</td>
<td>IHC</td>
<td>1:1000</td>
<td>ThermoFisher Scientific</td>
<td>Western blot analysis of Nodal expression in HeLa whole cell lysate and human recombinant Nodal fusion protein. Mouse mAb.</td>
</tr>
<tr>
<td>Nodal</td>
<td>sc81953</td>
<td>WB</td>
<td>1:1000</td>
<td>Santa Cruz</td>
<td>Western blot analysis of Nodal expression in HeLa whole cell lysate and human recombinant Nodal fusion protein. Mouse mAb.</td>
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<tr>
<td>phospho-4EBP1 (S65)</td>
<td>9451</td>
<td>WB</td>
<td>1:1000</td>
<td>Cell Signaling</td>
<td>Rabbit pAb. Western blot analysis of extracts from 293 cells using 4E-BP1 Antibody #9644 and Phospho-4E-BP1 (Ser65) Antibody #9451. The cells were starved for 24 hours in serum-free medium and underwent a 1 hour amino acid deprivation. Amino acids were replenished for 1 hour. Cells were then either untreated (-) or treated with 100 nM insulin (+) for 30 minutes. Citations: 190.</td>
</tr>
<tr>
<td>phospho-RPS6 (S240/244)</td>
<td>2215</td>
<td>WB</td>
<td>1:1000</td>
<td>Cell Signaling</td>
<td>Rabbit pAb. Western blot analysis of extracts from 293 cells, untreated or treated with 20% FBS for the indicated time, using Phospho-S6 Ribosomal Protein (Ser235/236) Antibody #2211 and Phospho-S6 Ribosomal Protein (Ser240/244) Antibody #2215. Citations: 334.</td>
</tr>
<tr>
<td>Slug</td>
<td>9585</td>
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<td>1:1000</td>
<td>Cell Signaling</td>
<td>Rabbit Monoclonal Antibody Western blot analysis of extracts from A204, SKME1, and NIH/3T3 cells using Slug (C19G7) Rabbit mAb. Citations: 203.</td>
</tr>
<tr>
<td>Snail</td>
<td>3879</td>
<td>WB</td>
<td>1:1000</td>
<td>Cell Signaling</td>
<td>Rabbit mAb. Western blot analysis of extracts from HCT116, HeLa, NIH/3T3, Rat2, and COS cells using Snail (C15D3) Rabbit mAb. Citations: 209.</td>
</tr>
<tr>
<td>Sox</td>
<td>3579</td>
<td>WB</td>
<td>1:1000</td>
<td>Cell Signaling</td>
<td>Rabbit mAb. Western blot analysis of extracts from NTERA2 and NCCIT cells using Sox2 (D6D9) XP® Rabbit mAb. Citations: 98.</td>
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<tr>
<td>Total RPS6</td>
<td>2217</td>
<td>WB</td>
<td>1:1000</td>
<td>Li-cor</td>
<td>Rabbit pAb. Western blot analysis of extracts from HeLa, NIH/3T3, PC12 and COS cells using S6 Ribosomal Protein (5G10) Rabbit mAb. Citations: 683.</td>
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<td>Twist</td>
<td>ab50581</td>
<td>WB</td>
<td>1:1000</td>
<td>Abcam</td>
<td>Rabbit pAb. Twist detected via western blot in 293T WT and 293T-Twist transfected cell lysate. Twist staining in murine brain tissue by immunohistochemistry. Citations: 52.</td>
</tr>
<tr>
<td>β-Actin</td>
<td>4970</td>
<td>WB</td>
<td>1:1000</td>
<td>Cell Signaling</td>
<td>Rabbit mAb. Western blot analysis of cell extracts from NIH/3T3, HeLa, PAE, and A431 cell lysates using beta-Actin (13E5) Rabbit mAb. Product citations: 1102.</td>
</tr>
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### Supplemental Table 3: G Blocks Used in Luciferase Reporter Assay Construction.

<table>
<thead>
<tr>
<th>gBlock Sequences:</th>
<th>Nanog_350</th>
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<td>ATATATTCGGCTCTCAACACCGAGCGACCTCGATAAGCGTTGACACAATGGGACAGGGAGCGGGGGATGGGGGAATTCAGCTCAGGCTTTTATGCAAAGACCCCCTTCTGCAAAGAAC</td>
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</tr>
</tbody>
</table>

**Note:** The above table lists the sequences of G Blocks used in the construction of luciferase reporter assays. Each row represents a different G Block, with the sequence details provided. The table includes sequences for Nanog, Snail, Nodal, and Nodal ALT. Each sequence is a string of nucleotides that are part of the G Blocks used in the construction process.
a SUM149

DMSO

4E-BP1

4E-BP1-P

INK

4E-BP1

4E-BP1-P
PDX401

DMSO

4E-BP1

4E-BP1-P

INK

4E-BP1

4E-BP1-P
PDX574

DMSO

4E-BP1  4E-BP1-P

INK

4E-BP1  4E-BP1-P
Supplemental Figure 3: Phosphorylated 4E-BP1 Abundance is Highly Variable Between Tumours and Models.

Immunohistochemically stained serial sections of SUM149 (a), PDX401 (b), and PDX574 (c) tumours treated with vehicle (DMSO) or INK128 (30mg/kg) were probed for total 4E-BP1, and phosphorylated 4E-BP1 (brown). The amount of 4E-BP1-P is highly heterogeneous. Images of serial sections of three individual tumours are present for each model to show the variability in the extent to which 4E-BP1 is phosphorylated.
Curriculum Vitae

Education

PhD Candidate Anatomy and Cell Biology
University of Western Ontario, London, Ont.
September 2009

Bachelor of Science – Honours Biology – Cooperative Education
University of Waterloo, Waterloo, Ont.
September 2003-August 2008
Specialization in Molecular Biology and Biotechnology
Dean’s Honours List Distinction

Work Experience

2008-2009 University of Waterloo – Student Life Office Waterloo, Ont
Coordinator for Graduate and Graduating Students
• Created resources for students seeking careers in academic professions.
• Established new training and transition programs to better prepare student for success beyond academic life.
• Managed mid and large-sized teams in the organization and execution of careers seminars and academic conferences.

2007 Public Health Agency of Canada Guelph, Ont
Salmonella Research Laboratory - Laboratory Technician
• Responsible for designing and testing of novel microarray techniques for serotyping Salmonella to single nucleotide resolution
• Developed a novel multiplex protocol for the genetic identification of Salmonella O-antigen genes
• Gained proficiency with genetic analysis software including the Lasergene Suite and Kodon Chromosome and Sequence Analysis Software

2005 Defense Research & Development Canada Toronto, Ont
Immuno-inflammatory Research Laboratory - Laboratory Technician
• Created cytokine, reactive oxygen species and adhesion protein flow cytometry identification protocols for assessment of hypertonic saline administration in patients
• Tested blood for major and minor derivatives of leukocytes to efficiently characterize immune response
• Produced comprehensive compendiums of literature pertaining to sepsis and immune regulation

2005 Agriculture and Agri-food Canada London, Ont.
Office of Biotechnology - Biotechnology Assistant Officer
• Routinely completed scientific tasks such as high throughput PCR, electrophoresis, DNA finger printing, northern blots as well as DNA and protein purification
• Used microcosm growth condition experiments to accurately and consistently identify bacteria with unique micro-climates
• Developed and implemented a quantitative real-time copy number PCR protocol for the enumeration of enteric bacteria for environmental samples

Publications:


Awards:

- Cancer Research Institute of Northern Alberta, Poster Presentation Award
- Anatomy and Cell Biology, Research Day Poster Award
- CaRTT-STP Scholarship, University of Western Ontario
- Schulich Graduate Scholarship, University of Western Ontario
- Dean's Honour List Distinction, Faculty of Science, University of Waterloo