# Assessment of the cell cycle proteins Cdc7 and PCNA as markers of colon carcinogenesis in obese and lean rats

by

Katherine Jean Wood

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis,including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

# Abstract

Obesity increases the risk of colon cancer as well as the expression of many cancer markers, ostensibly due to the interaction between insulin resistance and adipocyte production of hormones, mitogens and cytokines which collaborate to enhance proliferation signaling and impair the DNA damage response. Cdc7 and PCNA are both proteins involved in the DNA damage response as well as DNA replication. Both have also been shown to be upregulated in human tumours. To assess Cdc7 and PCNA roles during the DNA damage response in obese and lean animals, we administered azoxymethane (AOM), a colon-specific carcinogen, to obese and lean rats. Cdc7 and PCNA levels in colonic mucosal protein extracts from obese Zucker rats were compared with those from their lean counterparts. Significant differences were seen between lean and obese animals 3 hours post-AOM (lean Cdc7 levels > obese Cdc7 levels) and 24 hours post-AOM (lean PCNA levels > obese PCNA levels). This result suggests an impaired checkpoint response in obese animals relative to lean animals and supports a previously reported early role for Cdc7 in the checkpoint signaling cascade relative to a later role of PCNA in DNA damage repair. At the time tumours appeared (32 weeks post-AOM), colonic mucosal Cdc7 levels of obese rats exceeded that of their lean counterparts, suggesting that the obese metabolic environment causes upregulation of Cdc7 in obese rat epithelia. Cdc7 and PCNA levels were then compared between tumours and mucosa in obese and Sprague Dawley rats. Tumour Cdc7 levels were upregulated relative to mucosal levels in more samples than tumour PCNA levels, suggesting Cdc7 may be a more sensitive tumour marker. No significant differences in Cdc7 levels were seen between obese tumours and mucosa, likely due to elevation of obese mucosal Cdc7 levels. However, Sprague Dawley (non-obese) rats showed significantly higher Cdc7 and PCNA levels in tumours than mucosa, consistent with previous studies in human tissues. These results suggest that Cdc7 may be a more sensitive tumour marker than PCNA, but that its utility as a biomarker of colon cancer is dependent on the metabolic state (leanness) of the individual.

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***Nita** – I am <u>not</u> good at everything. That's you. :)*

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*To my gorgeous, wonderful family: Peter, Candace, Kyle, Alexander & Julie, without whose love and support this document, and the rest of my life, would be blank pages.*

*To Dad...wish you could have been here to see the wedding, the little babies, and my graduation...*

*To Mom, for her shining example.*

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# List of abbreviations

| | |
|---|---|
| ACF | aberrant crypt foci |
| And-1 | acidic nucleoplasmic DNA-binding protein 1, also known as WDHD1 |
| AOM | azoxymethane |
| ASK | associated with S-phase kinase (mammalian homologue of Dbf4) |
| ATM | ataxia telangiectasia mutated |
| ATP | adenosine triphosphate |
| ATR | ATM- and Rad 3-related |
| Cdc7 | cell division cycle protein 7 (S-phase kinase) |
| Cdc45 | cell division cycle protein 45 (accessory factor for MCM) |
| Cdk | cyclin-dependent kinase |
| CEA | carcinoembryonic antigen |
| Chk1 | checkpoint kinase 1 |
| Chk2 | checkpoint kinase 2 |
| CRC | colorectal cancer |
| DNA | deoxyribonucleic acid |
| DSB | double-stranded break |
| FOBT | faecal occult blood test |
| GINS | go ichi nii san, Japanese words for Sld5-Psf1-Psf2-Psf3, its subunits |
| Ki-67 | Kiel-67 (named after city in which antibody was first made) |
| MAM | methylazoxymethanol |
| MCM | minichromosome maintenance complex |

| | |
|---|---|
| Mcm1-10 | minichromosome maintenance proteins 1-10 |
| OL | quotient of means, obese/lean |
| ORC | origin recognition complex |
| PCNA | proliferating cell nuclear antigen |
| PCR | polymerase chain reaction |
| PIKK | phosphatidylinositol 3-kinase-related kinase |
| Psf1-3 | partner of Sld5, 1-3 |
| Rb | retinoblastoma protein |
| RFC | replication factor C |
| RNA | ribonucleic acid |
| Sld5 | synthetically lethal with Dpb11-5 |
| SSB | single-stranded break |
| SUMO | small ubiquitin-like modifier |

*"Stay curious."*

- *Gil Grissom, from CSI: Crime Scene Investigation*

# Chapter 1. Introduction

## 1.1 Overview

In Canada, cancer of the colon is the second leading cause of cancer death in males and the third leading cause of cancer death in females, accounting for approximately 12 percent of the total cancer deaths in both genders (Canadian Cancer Society's Steering Committee, 2009). The importance of early detection in optimizing prognosis and minimizing the cost of this disease has led to the discovery of a plethora of tumour markers. Protein tumour markers currently include replication factors such as proliferating cell nuclear antigen (PCNA), cyclin D1, cyclin E, Mcm2 and Cdc7 (Gould Rothberg et al., 2009; Kwee & Kwee, 2008; Semple & Duncker, 2004; Srivastava et al., 2001; Velmurugan et al., 2008). Variability amongst cancers coupled with the inconsistency of individual markers in accurately detecting cancer fuels the search for new candidates. Since cancer cells utilize a dysregulated DNA damage response to survive their increased mutation rate, replication factors that are also involved in DNA damage response may have increased likelihood of overexpression in cancer cells. Recent studies have revealed a novel, active role of the DNA replication kinase Cdc7 in checkpoint signaling (Kim et al., 2008; Tenca et al., 2007). Similarly, the replication fork component PCNA has known roles in DNA damage repair (reviewed in Moldovan et al., 2007). In this work, levels of Cdc7 and PCNA protein were assessed to determine if they mirrored their sequential involvement in DNA damage response immediately following carcinogen challenge in a rat model of colon carcinogenesis. Further, previous studies demonstrating impaired DNA damage

response and enhanced cellular proliferation in obese animals (Jaiswal et al., 2000; Mena et al., 2009; Zeng et al., 2008) suggested that obese pathophysiology could affect the robustness of the DNA damage response and the ability of both Cdc7 and PCNA to discriminate between tumour and non-tumour tissues. The performance of Cdc7 and PCNA as tumour markers in obese animals was assessed using obese, lean and Sprague-Dawley rat models of AOM-induced colon cancer.

## 1.2 Colon cancer

### 1.2.1 Tumourigenesis in the mammalian colon

The mammalian colon consists of three layers: the mucosa, which lines the interior (lumen) of the colon and contains primarily epithelial cells; the submucosa, which lies radially outside of the mucosa and is comprised of connective tissue; and the muscularis, the outermost muscular lining of the colon responsible for advancement of the feces. CRC is characterized by uncontrolled proliferation of colonic or rectal epithelial cells (Shibata, 2006; Vogelstein & Kinzler, 2004). Normal colonic mucosal epithelial cells begin as epithelial stem cells at the base of crypts in a region known as a niche (Shibata, 2006) (Figure 1). Mesenchymal cells beneath the basement membrane at the base of the crypt signal stem cells to remain undifferentiated. Stem cell progeny which leave the niche differentiate, migrate up the sides of the crypt towards the lumen, and eventually die and are sloughed off in the feces (Yen & Wright, 2006). DNA damage causes hyperproliferative changes in crypt architecture resulting in aberrant crypts (Bird & Good, 2000). Clusters of aberrant crypts known as aberrant crypt foci (ACF) (Figure 2) are identified by their abnormal morphology relative to nearby crypts:

they have enlarged, sometimes elongated luminal openings, thicker epithelial lining, and a well-defined pericryptal zone (Bird, 1987; McLellan et al., 1991a). ACF arise in the colon following carcinogen exposure in a dose- and time-dependent manner (McLellan et al., 1991a), as tumours do.

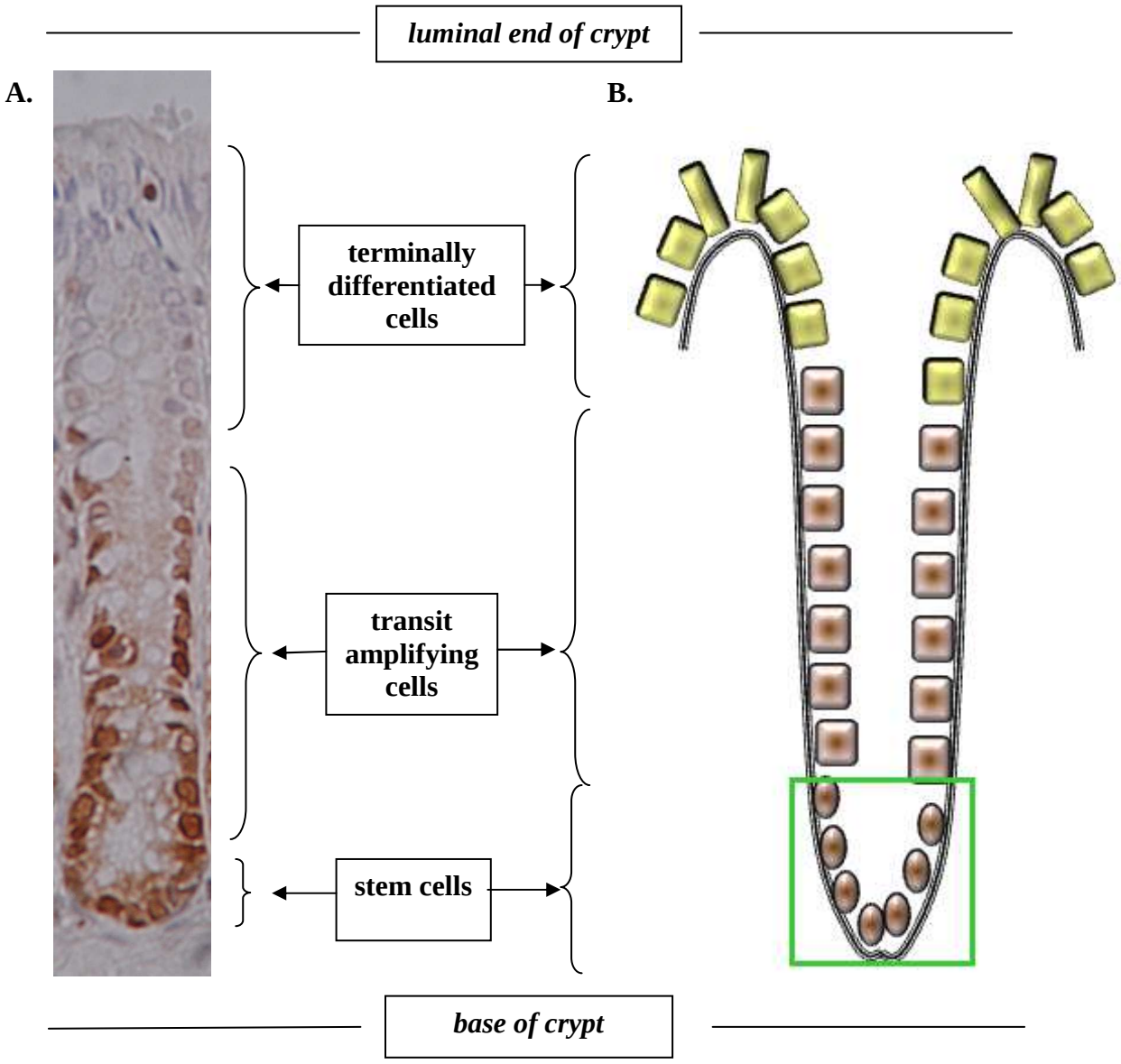



**Figure 1. Photograph (A) and cartoon rendering (B) of a colonic crypt immunostained to show proliferating cells.** Stem cells proliferate at the crypt base exclusively in the niche (round cells boxed in green in (B)). Cells which leave the niche divide a finite number of times as they migrate up the crypt wall toward the luminal end of the crypt. Eventually, they cease replicating, die, and are sloughed off. *Cartoon adapted from Shibata, 2008, Fig. 1.*

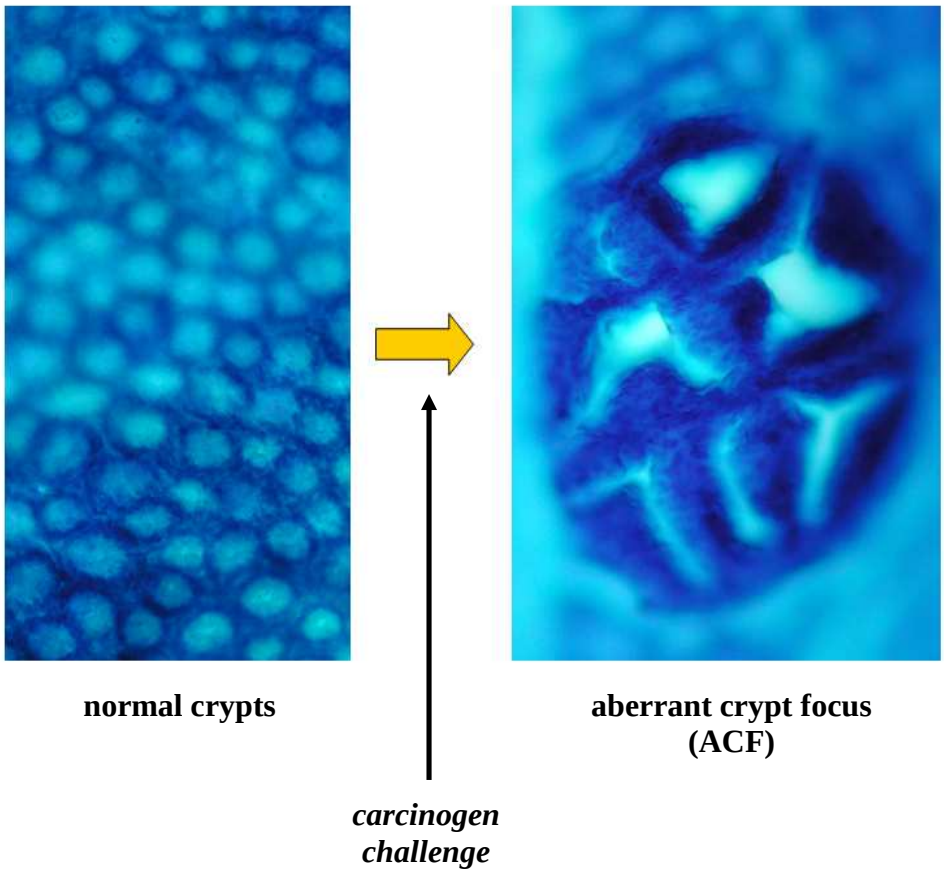



**Figure 2. Colonic crypt morphology is altered in response to DNA damage.** *Left panel:* Methylene-blue stained mucosa viewed from the luminal side contains crypts of uniform size and shape. *Right panel:* The same stain and view of an aberrant crypt focus demonstrates the differences in architecture of the crypts brought about by carcinogen challenge. Note that the focus protrudes into the lumen, and the luminal opening of each crypt of the focus is irregularly shaped, particularly in comparison to the normal crypts which can be seen in the background. *Magnification: 400x.*

Most ACF either cease to progress or eventually revert to normal tissue (Bird, 1995; Bird & Good, 2000). ACF can be classed according to the number of aberrant crypts involved in a particular focus; this feature of ACF is known as *crypt multiplicity*. Dysplastic ACF and ACF of high crypt multiplicity are thought to be particularly likely to progress to CRC (Gupta et al., 2007; Magnuson et al., 1993; Takayama et al., 2005). ACF that progress to CRC first become adenomatous polyps (benign tumours) which can be distinguished from carcinoma by growth confinement to the mucosal layers. Dysplastic cells in polyps have acquired additional mutations which confer a selective advantage which causes them to proliferate more effectively than other cells in the same crypt (Vogelstein & Kinzler, 2004), a phenomenon known as clonal selection. Additional mutations in polyp cells can lead to the selection of cell clones which are not restricted to the mucosal layers, known as neoplastic cells, resulting in carcinoma (malignant tumour) (Figure 3). Only a fraction of polyps have malignant potential to invade the muscular layers beneath the mucosa (Srivastava et al., 2001).

Colon carcinoma can be due in part to germline mutations which confer a highly increased risk for cancer development (hereditary CRC) or can arise solely due to somatic mutations (sporadic CRC) (Jass, 2002). Over 70% of CRCs are sporadic in nature (Benson, 2007), making models of sporadic CRC such as AOM-induced colon cancer a relevant tool in the search for useful cancer biomarkers.

### 1.2.2 Early detection of colon cancer

Early diagnosis of colon cancer improves the 5-year survival rate by 25-80% (Turnbull et al., 1997). Current detection and screening methods include colonoscopy, digital (with the fingers) rectal exam, barium enema, and the faecal occult blood test (FOBT)

(Beckman-Coulter). Since non-invasive methods are desirable for routine screening due to convenience, cost, comfort and reduced health risk to the patient (Ransohoff, 2002), the FOBT is the screening method of choice, with recent advances indicating higher sensitivity of immunochemical detection of hemoglobin over tests based on the chemical guaiac (Gimeno-Garcia et al., 2009). The presence of hemoglobin in the stool is, however, not perfectly correlated with cancer presence, as other diseases can also lead to colorectal bleeding. Also, polyps and even some carcinomas bleed intermittently and are therefore difficult to detect by this method alone (Turnbull et al., 1997). New detection targets are therefore needed to increase sensitivity of fecal testing.

Detection of molecules preferentially expressed in cancer cells relies on the favoured presence of polyp and cancer cells in stool. Polyp cells and carcinoma cells are non-apoptotic and are sloughed off at a greater rate than normal colonic epithelial cells (Loganayagam, 2008). This feature has been exploited to detect mutated DNA in feces of cancer patients; however, cost prohibits the routine use of DNA screening (Huerta, 2008). Since immunochemical methods have been successfully used to detect hemoglobin in feces (Gimeno-Garcia et al., 2009), protein biomarkers of CRC could become supplementary immunochemical targets for the FOBT. Detection of these new targets in cancer cells constitutively shed in feces could increase the sensitivity of the FOBT and regress the stage at which the cancer is detected at less cost than DNA screening. Earlier detection would then cause a concomitant reduction in morbidity and mortality due to CRC.

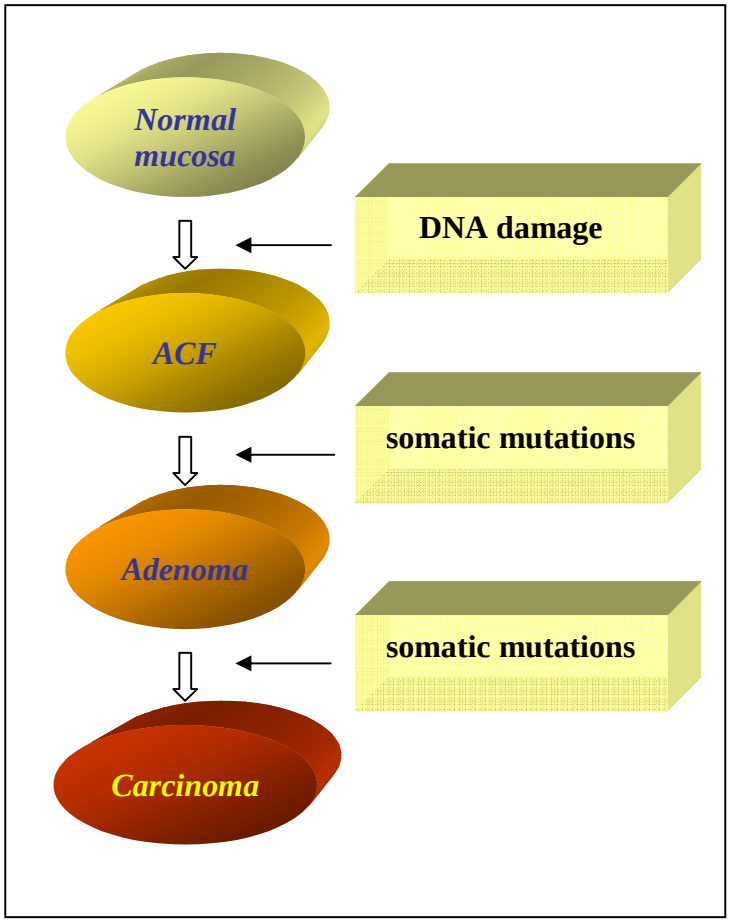



**Figure 3. Somatic mutations cause the progression of colon cancer.** DNA damage converts normal colonic crypts to hyperplastic ACF. Subsequent somatic mutations confer a selective advantage on a subset of ACF, which then become benign growths known as adenomas or polyps. Cells in some adenomas sustain further somatic mutations which allow the growth to invade neighbouring tissue, at which point the lesion is called a carcinoma. *Adapted from Fearon & Vogelstein, 1990.*

## 1.3 Protein markers of colon cancer

Proteins identified to date as markers of colon carcinogenesis lie in cellular proliferation pathways, DNA repair pathways, apoptosis pathways or vascularization pathways, congruent to features of colon cancer (Vogelstein & Kinzler, 2004). In high-risk populations, e.g. those with a first-degree relative diagnosed with colon cancer, candidate protein markers found in stool include immune-associated calprotectin, lactoferrin, and lysozyme, and serum proteins such as alpha-1-antitrypsin, albumin, and transferrin (Loganayagam, 2008). Proteins previously shown to be upregulated in tumours include: Ki67, a ribosome assembly factor (MacCallum & Hall, 2000) whose expression correlates with cellular proliferation (Scholzen et al., 2002) and which has been used as a biomarker in colon cancer (Fernandez-Cebrian et al., 2007); PCNA, a DNA replication fork component also upregulated during enhanced cellular proliferation and in tumours (reviewed in Semple & Duncker, 2004); and carcinoembryonic antigen (CEA), a cell adhesion protein which is only expressed in large quantities during fetal development and in tumour cells (Duffy et al., 2007). All tumour markers evaluated to date lack specificity, sensitivity or both, and therefore panels of markers are preferred (Duffy et al., 2007). The diversity of expression profiles in different cancers even within a tissue type also highlights the need for new biomarkers which could possibly be included in such panels.

## 1.4 Nucleic acid markers of colon cancer

Proteins are not the only molecular biomarkers of colon cancer. There are numerous gene mutations which are known to occur during the ACF-adenoma-carcinoma sequence of

events (Figure 3) and can provide targets for PCR amplification. Transformed cells shed in the feces contain mutated DNA sequences which can be detected via PCR (Duffy, 1995). Products of genes mutated in cancers participate in many cellular pathways, including apoptosis, DNA replication, mismatch repair, and tissue vascularization (Huerta, 2008), and their levels as well as levels of downstream targets may be altered as a result. However, while a single mutation can be inexpensive to detect, colorectal tumours display on average 4-6 out of the hundreds of possible mutations (Fearon & Vogelstein, 1990), rendering mutational screening more costly than originally thought. Also, a functional protein product must be translated from an mRNA transcript for upregulation to have an effect, and this translation can be modulated post-transcriptionally by the cell, leading to discrepancies between mRNA levels and phenotypic changes. These discrepancies render ideal target prediction much more difficult.

Non-protein markers of cellular proliferation have also included $^{3}$H-thymidine, a radioactively-labelled nucleotide, and BrdU (bromodeoxyuridine), a thymidine analogue to which antibodies are commercially available (Alberts et al., 2002; McLellan et al., 1991a). In the presence of one of these thymidine substitutes, replicating cells will incorporate it in place of some thymidine residues during replication. The level of detected thymidine substitute can therefore theoretically be used as a relative measure of replication activity. $^{3}$H-thymidine has been used to assess proliferative characteristics of ACF, CRC cells and xenografts in studies of chemoprevention, diet effects and treatments as well as to contribute to prognosis predictions in post-resection biopsy (Costa et al., 1997; McLellan et al., 1991a; Tangpricha et al., 2005; Wang et al., 2007; Xie et al., 2006). However, since $^{3}$H-thymidine has recently been

shown to induce cell cycle arrest and apoptosis in cells, its validity in assessing DNA replication rates is questionable (Hu et al., 2002).

BrdU has also been used to assess cellular proliferation rates in ACF-containing mucosa (Magnuson et al., 1994; Sutherland & Bird, 1994). When BrdU is applied by injection one hour prior to termination of previously AOM-challenged Sprague Dawley rats, resulting ACF appear to contain more BrdU-labelled cells than normal crypts (Magnuson et al., 1994), but there was no significant difference seen between the number of BrdU-labelled cells per 100 cells between those two crypt types (Magnuson et al., 1994; Sutherland & Bird, 1994). These results indicate that ACF contain more cells overall than normal crypts, but the same proportion of them are actively cycling. This is consistent with the morphologically larger size of ACF at the tissue level, while not being particularly useful in supporting the prognostic value of ACF. It has been suggested that circadian rhythms may be a determining factor in cell proliferation and migration in colonic crypts, since functional “clock genes” are expressed there (Hoogerwerf et al., 2007). Recent research has suggested that stem cells increase in number as part of carcinogenesis in crypts (Boman et al., 2008)

## 1.5 DNA replication and the cell cycle

The utility of PCNA as a tumor marker was discovered prior to the elucidation of its role in DNA replication (Chan et al., 1983; Mathews et al., 1984). Since then, a much more detailed model of DNA replication in which many newly elucidated proteins play a regulatory role has been constructed (Bell & Dutta, 2002) (Figure 4). The utility of many DNA replication factors, particularly those involved in replication initiation, as more sensitive tumour markers than traditional ones such as PCNA and Ki67 is currently being revealed (see Semple & Duncker, 2004 for a review). An overview of factors involved in DNA replication is presented below.

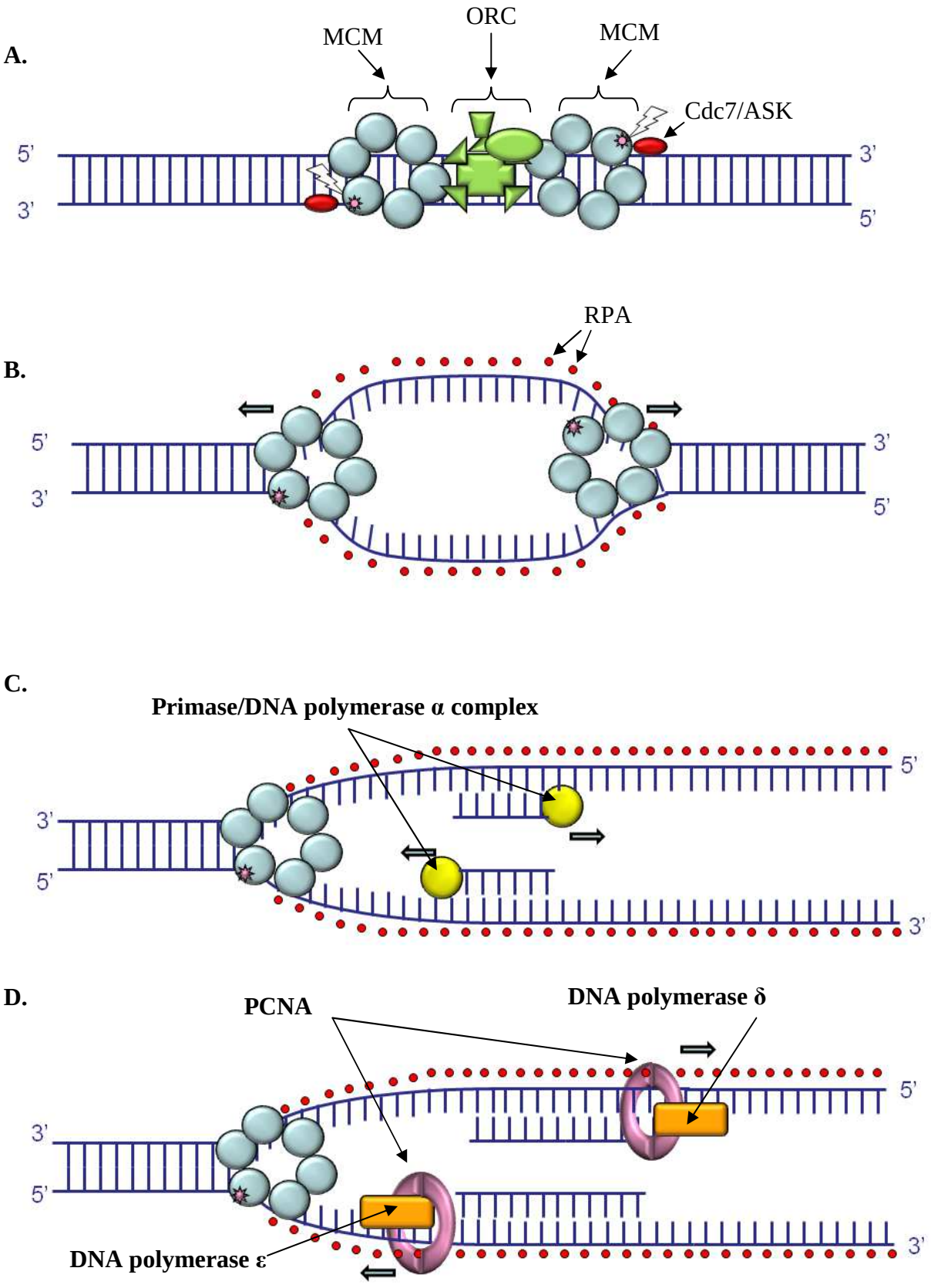
DNA replication occurs during S phase of the cell cycle (Figure 5). The accuracy of DNA replication prior to cell division is confirmed during G2 phase; nuclear (mitosis) and cytoplasmic division (cytokinesis) occur during M phase; and most factors necessary for the next round of DNA replication are assembled during G1 phase. Progression of each phase of the cell cycle is controlled via the regulation of activating kinases (Bell & Dutta, 2002).

DNA replication is initiated by mitogen signaling which induces the expression of immediate early genes such as fos, jun & myc (Masai et al., 2005). Immediate early gene products lead to expression of cyclin D which activates Cdk4 enabling it to phosphorylate the retinoblastoma (Rb) protein (Masai et al., 2005). Phosphorylation of Rb releases previously bound E2F transcription factor (Masai et al., 2005). Unbound E2F induces the transcription of replication factors such as the S-phase cyclins E and A, the Cdc7-activating protein, ASK (associated with S-phase kinase), and subunits of MCM helicase (Kim et al., 2003; Masai et al., 2005). The origin recognition complex (ORC), in cooperation with other factors,

effects the loading of MCM helicase onto origins of replication during G1 phase in an ATP-dependent event (Bell & Dutta, 2002). Activation of MCM, the final step in late G1 before DNA replication can begin, is accomplished via phosphorylation of MCM subunits by Cdc7/ASK, the activity of which has been demonstrated *in vitro* to be enhanced by prior MCM phosphorylation by Cdk2/cyclinE (Kim et al., 2003; Masai et al., 2000; Masai et al., 2005). Changes in MCM conformation then allow recruitment of Cdc45 and GINS forming a complex known as CMG which is capable of processive unwinding of DNA (Sclafani & Holzen, 2007). In budding yeast, CDKs additionally promote DNA replication initiation by phosphorylating Sld2 and Sld3, both of which are required for initiation (Zegerman & Diffley, 2007). Phosphorylation of MCM subunits by S-phase CDKs negatively regulates re-initiation by blocking MCM loading once activation is accomplished (Masai et al., 2005; Sclafani & Holzen, 2007).

Unwinding of origin DNA by the CMG complex produces single-stranded DNA which is prevented from re-annealing by the single-stranded DNA-binding protein replication protein A (RPA) (Moldovan et al., 2007). A DNA-dependent RNA polymerase called primase, complexed with DNA polymerase α, is recruited to DNA by CMG-associated factors Mcm10 and And-1 (Zhu et al., 2007). Primase synthesizes a short stretch of RNA complementary to origin DNA which is then extended with DNA complementary to the next ~30 nucleotides by DNA polymerase α (Stillman, 2008). The 3′ end left by termination of synthesis by DNA polymerase α attracts replication factor C (RFC) (Moldovan et al., 2007). RFC, in cooperation with RPA, acts as a clamp loader to load PCNA onto the single strand (Sclafani & Holzen, 2007). PCNA is a homotrimeric sliding clamp ring responsible for tethering DNA polymerases to the template and thus increasing their processivity during

**Figure 4. Initiation of DNA replication. A.** DNA replication initiation begins with binding of ORC and its associated factors (green) to origins of replication. These factors then effect the loading of MCM helicase (light blue) onto double-stranded DNA. MCM is activated by phosphorylation by Cdc7/ASK (red oval). Pink star indicates the site of MCM phosphorylation (Masai et al., 2005). **B**. Activated MCMs, in concert with Cdc45 and the GINS complex (not shown), begin to unwind origin DNA in a bidirectional fashion (Sclafani & Holzen, 2007). RPA (red circles) binds the single-stranded DNA accumulated behind the helicase to prevent re-annealing (Moldovan et al., 2007). Block arrows indicate direction of unwinding. **C.** Short (20-22 nt) primers are laid down by the primase/DNA polymerase α complex (yellow) (Moldovan et al., 2007). Block arrows indicate direction of primer synthesis. **D.** The 3′ end of primers is the site where PCNA (purple) is loaded Moldovan et al., 2007). PCNA then recruits the replicative DNA polymerases ε (leading strand, shown at bottom) and δ (lagging strand, shown at top) (orange rectangles) and elongation begins (Moldovan et al., 2007). Block arrrows indicate direction of elongation.


A.
MCM
ORC
MCM
Cdc7/ASK
5'
3'
3'
5'
B.
RPA
5'
3'
3'
5'
C.
Primase/DNA polymerase α complex
3'
5'
5'
3'
D.
PCNA
DNA polymerase δ
3'
5'
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DNA polymerase ε

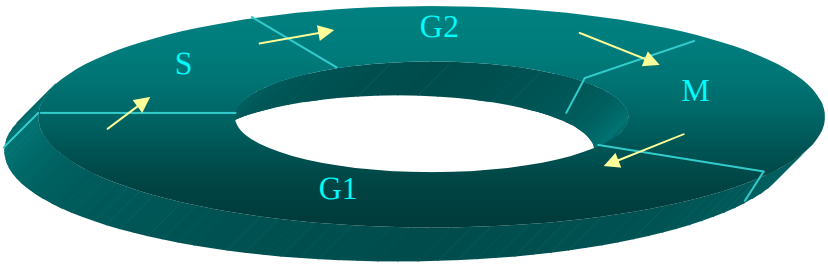



**Figure 5. Phases of the cell cycle.** The cell cycle consists of four phases: G1, S, G2, and M. Transition between the respective phases is accomplished via regulation of kinase activation (Bell & Dutta, 2002). DNA replication occurs during S phase, but factors involved in its inititation can be loaded onto chromatin as early as late M phase (Masai et al., 2005).

DNA synthesis (Moldovan et al., 2007). Once PCNA has attracted the replicative polymerases to DNA, elongation is effected by the concerted effort of the complete replisome, including MCM helicase, PCNA, and the DNA polymerases. Thus, Cdc7 functions as an essential regulator of S-phase entry and PCNA participates as part of the replication complex during the elongation process once S-phase is initiated.

## 1.6 DNA damage detection and repair

Damage to the DNA template can lead not only to defects in gene products but also to errors in replication which can jeopardize genome integrity. Tumours detected through the use of biomarkers arise as a major consequence of the failure of the DNA damage response to prevent chromosomal instability and mutation. Because DNA damage can pose a serious threat to cellular viability, cells have evolved multiple mechanisms to detect and repair such damage (Branzei & Foiani, 2008; Kastan & Bartek, 2004; Moldovan et al., 2007). DNA damage can be caused by ionizing radiation, errors in replication, or exposure to chemical carcinogens (Kastan & Bartek, 2004). Popular chemical carcinogens used in rat models of colon cancer are typically precursors of the DNA alkylating agent methylazoxymethanol (MAM), and include dimethylhydrazine and its metabolite AOM (Sohn et al., 1991). DNA alkylation resulting from carcinogen challenge is associated with sister chromatid exchange, gene mutations and chromosome rearrangements in mammals. The size of the alkyl adduct determines which repair mechanism will be employed (Kastan & Bartek, 2004). Simple methyl adducts can often be excised by an enzyme called methylguanine DNA methyltransferase (Sancar et al., 2004). More bulky DNA alkylations cause fork stalling and

trigger more complex DNA repair mechanisms such as base excision repair (Sancar et al., 2004). Furthermore, extensive methylation or alkylation can lead to DNA crosslinking and DNA double-stranded breaks (DSBs) (Sancar et al., 2004). Sensing of DNA damage during replication involves pathways known as cell cycle checkpoints (for reviews, see Branzei & Foiani, 2005; Branzei & Foiani, 2009; Kastan & Bartek, 2004; Sclafani & Holzen, 2007).

There are two cell cycle checkpoint pathways, named after the PI(3)K-like kinases (PIKKs) associated with their initiation: the ataxia telangiectasia mutated (ATM) pathway, which is activated in response to double-stranded break damage, and the ataxia telangiectasia and Rad3-related (ATR) pathway, induced by fork stalling and single-stranded breaks (SSBs), including those SSBs which are formed as an intermediate product of DSB repair (Branzei & Foiani, 2008; Branzei & Foiani, 2009; Kastan & Bartek, 2004; Matsuoka et al., 2007). Of the two PIKKs, only ATR is essential for viability, due in part to its important roles in promoting fork stability and monitoring fork progression during normal replication (Kastan & Bartek, 2004). ATM and ATR exert their effects through the activation of effector kinases known as Chk2 and Chk1 respectively (Kastan & Bartek, 2004).

There is evidence that Cdc7 is involved in the ATR–mediated response to fork stalling in human cancer cells and mouse embryonic stem cells (Kim et al., 2008; Tenca et al., 2007). Damage-induced fork stalling causes polymerase to pause while helicase continues to unwind DNA, leading to extended stretches of exposed single-stranded DNA (Branzei & Foiani, 2005; Branzei & Foiani, 2009; Zegerman & Diffley, 2009). The presence of large stretches of single-stranded DNA serves as a signal to recruit ATR to stalled forks at which PCNA-associated Chk1 (Scorah et al., 2008) and the mediator protein claspin are normally bound (Zegerman & Diffley, 2009). Cdc7, which is active during perturbed S phase (Tenca et al.,

2007), phosphorylates claspin, activating it and thus enabling claspin-mediated Chk1 activation by ATR (Kim et al., 2008) (Figure 6). It is not known whether Cdc7 levels are increased in association with this function; however relatively constant levels of Cdc7 are present during the normal cell cycle (Masai et al., 2005), suggesting that short-term fluctuations in Cdc7 levels can be attributed to other functions such as DNA damage signaling. Chk1 activation then prevents the onset of mitosis, firing of late origins, and fork collapse (Kastan & Bartek, 2004; Kim et al., 2008).

Interestingly, checkpoint inhibition of late origin firing in yeast is accomplished in part by impeding Cdc7 activity through phosphorylation of the yeast ASK homologue, Dbf4 (Branzei & Foiani, 2005; Costanzo et al., 2003; Kastan & Bartek, 2004). ASK itself is an *in vitro* target of Chk1 kinase activity as well as interacting with Chk1 *in vivo* in human cells in response to UV irradiation, suggesting that ASK may be a target of Chk1 activity during checkpoint response (Heffernan et al., 2007). Since Cdc7 remains active following checkpoint initiation (Tenca et al., 2007), inibition of late origin firing may be accomplished simply by disengaging ASK from origins, while levels of active Cdc7 remain the same or increase due to upregulation of non-origin-bound Cdc7 in response to ATR signaling.

Sometimes, fork collapse cannot be averted by the ATR-Chk1 signal. In the event of fork collapse, PCNA associated with the replisome is modified by SUMO or ubiquitin (Branzei & Foiani, 2005; Moldovan et al., 2007). These modifications are thought to release DNA polymerase δ or ε from PCNA and increase its affinity for alternative DNA polymerases (Branzei et al., 2008; Moldovan et al., 2007), such as polymerases κ, ι, and η, each of which contains a unique pocket designed to accommodate a specific type of lesion (Moldovan et al., 2007). Following PCNA-mediated repair, it is not known whether PCNA is deubiquitylated

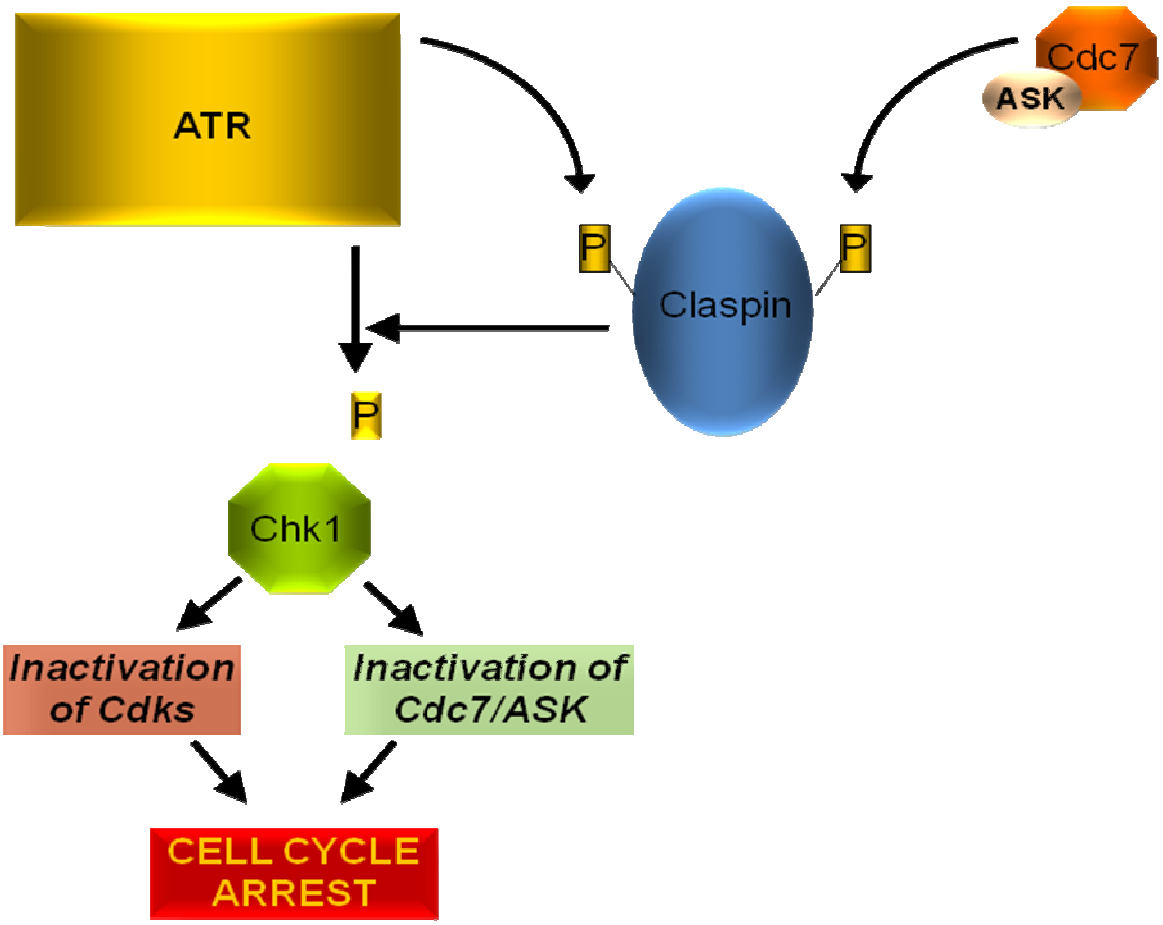



**Figure 6. Cdc7 is involved in claspin-mediated ATR signaling.** In response to fork stalling, ATR phosphorylates the mediator protein claspin. Recent evidence suggests that Cdc7/ASK also phosphorylates claspin as part of its activation (Kim et al., 2008). Activated claspin mediates the activation of Chk1 kinase by ATR (Kastan & Bartek, 2004). This effector kinase then acts on multiple substrates to arrest the cell cycle and initiate DNA damage repair processes (Kastan & Bartek, 2004).

or replaced by a new, unmodified moiety (Moldovan et al., 2007). If modified PCNA must be replaced, expression of PCNA is likely upregulated to make the substitution. In the event that the lesion is not passable by alternative polymerases, template switching may be initiated, also mediated by PCNA (Moldovan et al., 2007). Because PCNA-mediated repair processes follow fork collapse which follows ATR signaling, upregulation of PCNA to supply restarting forks should follow upregulation of Cdc7 during checkpoint initiation.

Sometimes, even the elegant DNA damage response machinery in the cell is insufficient to avert permanent changes in the DNA sequence. Depending on their location and extent, such mutations may cause no phenotypic changes; they may cause minor, benign phenotypic changes; they may cause the cell to become non-viable; or they may complete a set of previous mutations, resulting in a cancerous cell.

## 1.7 Cdc7 and PCNA as cancer biomarkers

There is some evidence that Cdc7 levels are increased in tumour cells relative to those in normal tissues (Bonte et al., 2008; see also Hess et al., 1998; reviewed in Semple & Duncker, 2004). Cdc7 was originally suggested as a potential cancer biomarker in mRNA studies of multiple tissue types and cell lines (Hess et al., 1998). Several other replication initiation proteins, including some MCM subunits, have previously been shown to be more sensitive markers of abnormal cells in some cancers than either PCNA or Ki67 (see Semple & Duncker, 2004 for a review). More recently, Cdc7 protein levels have been shown to be upregulated in human colon and breast cancer tissues and in multiple cancer cell lines (Bonte et al., 2008) as well as in melanoma (Clarke et al., 2009). Cdc7 additionally has prognostic value in assessing potential morbidity due to ovarian carcinoma (Kulkarni et al., 2009).

Small-molecule inhibitors of Cdc7 kinase activity are now being developed as novel cancer treatments, which have the advantage of both impeding replication initiation and obstructing the DNA damage response which cancer cells rely on to avoid lethal mutations (Menichincheri et al., 2009; Montagnoli et al., 2008; Vanotti et al., 2008).

PCNA has been ubiquitously employed as an immunohistochemical marker of cellular proliferation in most cancers, including hepatocellular carcinoma (Shen et al., 2008), gastric cancer (Li et al., 2008), and colon cancer (Baijal et al., 1998). Evidence has been presented which indicates that PCNA may not accurately reflect the early development of precancerous lesions. Sutherland and Bird (1994) investigated the effect of chenodeoxycholic acid (a bile acid purported to promote cancer) on the development of ACF as a result of AOM injection. The percentage of cells staining positive for PCNA ("labelling index") was the same or slightly higher in colons of animals not exposed to carcinogen, indicating that PCNA expression may actually be decreased in precancerous stages, although possibly not to an extent which is useful for detection. PCNA labelling index is also not independent of the presence of exogenous bile acids even without carcinogen challenge (Baijal et al., 1998), suggesting that the presence of PCNA may indicate pathologies not related to carcinogenesis. This may be due in part to upregulation of PCNA following some forms of successful DNA damage repair (reviewed in Moldovan et al., 2007) (see section 1.6).

## 1.8 Predisposition of overweight and obese animals to colon cancer

Obesity complicates the picture of cancer. Disorders associated with obesity such as hyperlipidemia, hyperglycemia, hyperinsulinemia, hypertriglyceridemia and diabetes all show

comorbidity with CRC (Campbell & McTiernan, 2007). Obesity-associated visceral abdominal fat is positively correlated with putative preneoplastic lesions (Takahashi et al., 2009). Obese animals are at greater risk than lean animals of developing tumours in response to AOM administration (Eskin et al., 2007; Raju & Bird, 2003; Weber et al., 2000).

Increased CRC risk in obese animals appears to be largely due to inhibition of DNA damage response coupled with enhanced cellular proliferation. Cytokines expressed by adipocytes promote colon carcinogenesis and inhibit DNA repair by enhancing inflammation and nitrosylating damage response enzymes (Jaiswal et al., 2000; Mena et al., 2009). Saturated fatty acids, which are much more plentiful in obese animals, have also been shown to inhibit the DNA damage response in untransformed cells, through unclear mechanisms (Zeng et al., 2008). Increased cellular proliferation signaling affects normal and tumour tissues causing tumours to grow faster in obese animals. Hyperinsulinemia and high blood glucose levels commonly seen in obese populations augment cellular proliferation in the colon (Mena et al., 2009). Tumour cells appear to induce expression of some cancer-promoting factors in neighbouring adipocytes and fibroblasts, leading to enhanced tumour growth in adipocyte-rich obese animals (Motrescu & Rio, 2008). Hence, obesity leads to impairment of the DNA damage response resulting in increased risk for mutation, and enhanced cellular proliferation signaling subsequently leads to augmented tumour growth.

#### 1.8.1.a Animal models of obesity

Increased risk for development of colon cancer has been demonstrated in both obese mice and obese rats (Ealey et al., 2008; Kobayashi et al., 2000; Raju & Bird, 2003). The Zucker obese (*fa/fa*) rat resulted from a spontaneous autosomal recessive mutation in a cross between Sherman rats and the 13M strain of *Rattus norvegicus* (Argiles, 1989; Bray, 1977).

Originally designated the *fa* (fatty) gene, the mutation was later discovered to be a loss-of-function mutation in the leptin receptor, causing a lack of sensitivity in the recessive homozygote to satiety signaling (Argiles, 1989). Together with its lean (*Fa*/*Fa* or *Fa*/*fa*) counterpart, the Zucker obese rat model has greatly facilitated studies of the etiology of obesity as well as the interaction between obesity and disorders such as hypertension (Dakshinamurti et al., 1998; Kanda et al., 2006; Kim et al., 1994; Namikoshi et al., 2008), insulin resistance (Deushi et al., 2007; Kiunga et al., 2004; Whaley-Connell et al., 2008; Zhang et al., 2007), hepatic steatosis (Deushi et al., 2007; Raju & Bird, 2006), and even erectile dysfunction (Wingard et al., 2007). The obese Zucker rat's inherent hyperinsulinemia, enhanced cellular proliferation and general metabolic syndrome are associated with increased risk for development of tumours (Koch et al., 2008; Raju & Bird, 2003; Raju et al., 2006; Weber et al., 2000). It is therefore a credible model to study the differences between obese and lean animals during colon carcinogenesis (Raju & Bird, 2003; Raju et al., 2006).

## 1.9 Objectives and hypotheses

The purpose of this work was to investigate the relationships between Cdc7 expression, PCNA expression, colon carcinogenesis and obesity in a rat model. The main objective of this research was to assess expression levels of Cdc7 and PCNA during early and late stages of colon carcinogenesis to determine their utility as markers of early and late events.

We specifically hypothesized that:

1. Since Cdc7 is involved in ATR checkpoint activation (Figure 6) and PCNA upregulation occurs following DNA damage repair (see section 1.6 in the Introduction), Cdc7 protein levels should peak prior to PCNA levels during the first 24 hours following AOM administration. Changes in Cdc7 levels are expected to be due to non-cell-cycle roles since Cdc7 levels are relatively constant throughout the cell cycle in normal cells.

2. As a result of increased proliferation signaling over their lean counterparts, Zucker obese rats should show elevated Cdc7 and PCNA levels in mucosa relative to lean by the time tumours appear.

3. Both Cdc7 and PCNA protein expression will be higher in Sprague Dawley rat tumours compared to normal-appearing mucosa, as shown previously in human mRNA studies for Cdc7 and multiple studies for PCNA.

To test these hypotheses, colonic tissues were collected for three different studies:

**Study 1**: Zucker obese and lean rats were used to model of the effect of obesity on Cdc7 and PCNA expression during colon carcinogenesis (see section 1.8). Animals were injected with the carcinogen AOM and tissues were analysed for Cdc7 and PCNA levels 3, 9 and 24 hours post-injection;

**Study 2**: Obese and lean rats injected with AOM were allowed to develop tumours. Tumours and normal-appearing colonic mucosa collected 32 weeks post-AOM were then assessed for Cdc7 and PCNA levels;

**Study 3**: Sprague Dawley male rats, an experimental model commonly used to study colon carcinogenesis, were injected with AOM. Colonic tumours were assessed for Cdc7 and PCNA levels and compared to those of normal-appearing colonic mucosa collected 32 weeks post-AOM.

# Chapter 2. Materials and Methods

## 2.1 Rat strains

Two strains of *Rattus norvegicus* were used during this study: Zucker, a genetically obese rat homozygous recessive for a nonfunctional leptin receptor, together with its lean (homozygous dominant/heterozygous) counterpart; and Sprague Dawley, a non-obese, common laboratory rat.

### 2.1.1 Zucker

Thirty-five obese (fa/fa) and forty lean (Fa/Fa or Fa/fa) seven-week-old female Zucker rats were acquired from Charles River Laboratory (Wilmington, MA). All animals were housed in wood-shaving-lined plastic cages with stainless straight wire lids designed to accommodate food and water delivery. Animals were allowed to acclimate to the animal housing facility (Biology Department, University of Waterloo) for 10 days, during which time they were fed standard lab chow (modified AIN-76A, Harlan Teklad). A uniform temperature of 22°C and humidity of 55% were maintained in the animal housing facility. Following acclimation, all but 12 (6 obese and 6 lean) were challenged with a single dose of AOM at 10 mg/kg, 24h, 9h, or 3h prior to termination (Figure 7). A reduced dose relative to that used for Sprague Dawley rats was delivered to the Zucker rats to allow for possible increased toxicity of AOM in Zucker obese rats (Eskin et al., 2007). All Zucker obese and lean rats involved in Study 1 (see Objectives and hypotheses) were terminated concurrently. The remaining five obese and ten lean Zucker rats were terminated 32 weeks post-AOM challenge (Study 2).

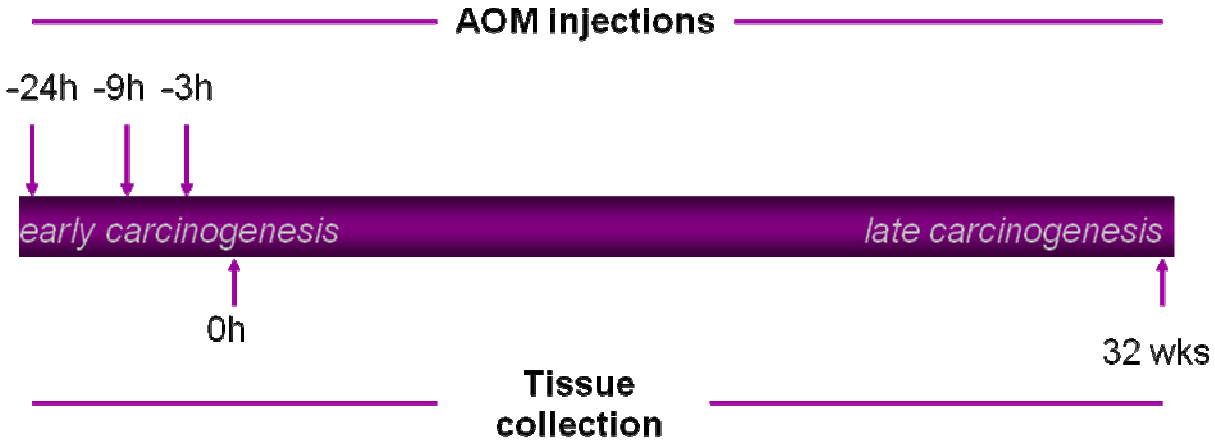



**Figure 7. Experimental design for Zucker studies (Study 1 and Study 2).** Groups of obese and lean rats were either left uninjected or injected with AOM 24h, 9h, or 3h prior to the first termination (0h). An additional group of rats of each obese phenotype were additionally injected with AOM at the -3h timepoint; these animals were terminated 32 weeks post-AOM. Tumours were excised from 32-week specimens, and all mucosa were collected. Tissue samples were snap-frozen in liquid nitrogen and stored at -80°C pending western blot sample preparation.

### 2.1.2 Sprague Dawley

Tissues from male Sprague Dawley rats terminated for a previous study (Begleiter et al., 2003) were used for Study 3. Six-week old male Sprague Dawley rats were previously appropriated from the University of Manitoba Central Animal Care Breeding Facility. The animals were originally fed a modified AIN-76A (American Institute of Nutrition-76A) powdered diet (Harlan Teklad) with 13% dextrose and 52% cornstarch, challenged with the colon-specific carcinogen AOM at 15mg/kg in two weekly doses, and terminated at 32 weeks post-challenge (Figure 8). Colons were previously removed, flushed with 0.9% saline and slit lengthwise. Mucosa was scraped away from muscularis, snap-frozen in liquid nitrogen, and stored at -80°C.

All animals were cared for according to the guidelines of the Canadian Council on Animal Care, and the experimental protocol was approved by the University of Waterloo Animal Care committee.

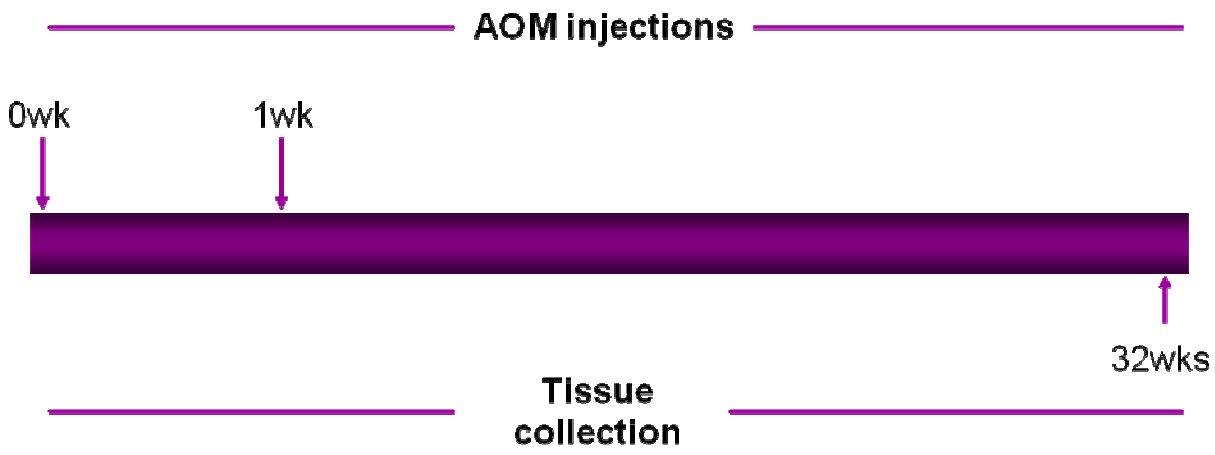



**Figure 8. Experimental design for Sprague Dawley study (Study 3).** Sprague-Dawley rats were given two injections of AOM one week apart. Animals were terminated 32 weeks later, tumours were excised and mucosa collected. All samples were snap-frozen in liquid nitrogen and stored at -80°C until samples were prepared for Western blot.

## 2.2 Tissue collection

Colons were removed, flushed and slit lengthwise as for Sprague Dawley rats. Tumours were excised from the mucosa using scissors and placed in labelled 1mL capped plastic tubes (Eppendorf). Mucosa was scraped away from muscularis and placed in similar labelled tubes. All tissues were snap-frozen in liquid nitrogen and stored at -80°C. Five mucosal samples were randomly chosen from the final ten lean Zucker rats for inclusion in Study 2.

## 2.3 Tissue homogenization and extraction of soluble proteins

Frozen mucosa and tumours were sliced thinly then chopped into fine pieces on a fresh Petri dish at 4°C using a clean razor blade. Each sample was then dropped into a labelled glass test tube containing 4mL modified RIPA buffer (50mM Tris; 1% NP-40; 0.25% sodium deoxycholate; 150mM NaCl; 1mM EDTA; 1mM NaF; 1mM sodium orthovanadate; 1mM PMSF) per gram of tissue, rounded up to the nearest 1mL. Test tubes were kept covered with Parafilm® (American National Can Company) when not in use. Tissues (still in buffer) were homogenized on ice using a Polytron® PT-2100 disperser (Kinematica) with a 12mm aggregate twice for 15 seconds each, cooling for at least 30 seconds in between. Resulting homogenates were then removed to labeled, capped 1mL Eppendorf tubes and spun at 4°C using a refrigerated centrifuge (Eppendorf 5415R) for 15 minutes at 16.1*g* to pellet the insoluble proteins and float the lipids. The clearest part of the liquid layer between the pellet and lipids was removed to a fresh labeled 1mL Eppendorf tube. A portion of each resulting

sample was assayed using the Bradford method to determine total protein concentration. On the basis of these concentrations, western blot samples were made up containing 50μg total protein using 1:1 protein extract:2X Laemmli buffer (Sigma).

## 2.4 Western Blot

Protein extracts from colonic tumour samples and samples from adjacent colonic mucosae of male Sprague Dawley rats treated with AOM and terminated after 32 weeks were analyzed by Western blot for the presence of Cdc7 and PCNA, with HeLa cell lysate (Santa Cruz) as a positive control, using established laboratory protocols.

Electrophoresis of samples prepared for western blot was accomplished using the equipment and methods of the Mini-Protean III and Mini Trans-blot systems (BioRad). Briefly, 8% polyacrylamide resolving gels with 6% stacking gels were loaded with one sample per lane, including one HeLa positive control (Santa Cruz) and at least one molecular weight marker (Sigma or Fermentas) for each gel and were electrophoresed at 120V for at least 90 minutes (until the dye front had progressed to near bottom of gel, and the marker for molecular weight closest to that of Cdc7 (64kDa) had migrated to the middle of the gel). Electrophoretic transfer to nitrocellulose membranes was then performed at 100V for one hour, using a Mini Trans-Blot® wet transfer unit (BioRad) and Buffer A (25mM Tris, pH 8.3; 192mM glycine, 20% methanol and 0.05% sodium dodecyl sulfate (SDS)).

Membranes were rinsed twice with distilled water to remove transfer buffer then immersed in 0.1% Ponceau S in 5% acetic acid for 1 hour to stain total proteins. Following Ponceau staining, membranes were removed to distilled water for 2-5 minutes to remove

excess stain and improve contrast between membrane-bound block alone and total cellular proteins. They were then drained by dabbing a corner on a Kimwipe and placed face down on a scan bed (Epson). To remove bubbles from between the membrane and the scan bed, the membrane was rolled gently with a glass rod. A digital image of the Ponceau-stained membrane was acquired at 300 dpi in colour. If protein lanes did not contrast sufficiently with background, the membrane was placed in a second trough of fresh distilled water, and the scan was repeated to ensure the clearest representation of the relative amount of total protein in each lane. Membranes were then washed twice for five minutes by gentle rocking in T-TBS wash buffer (0.02M Tris pH 7.5; 0.15M NaCl; 0.05% Tween-20) to remove remaining Ponceau stain and blocked in 25mL 5% skim milk in T-TBS for 1 hour at room temperature or overnight at 4°C. Membranes were then incubated with polyclonal rabbit Cdc7 antibodies (Santa Cruz) in 1% skim milk/T-TBS at a dilution of 1:200 (10mL per membrane) for 105 minutes at room temperature or overnight at 4°C, rocking gently. After three vigorous washes in T-TBS, horseradish peroxidase (HRP)-conjugated secondary antibody at a dilution of 1:3000 in 1% skim milk/T-TBS was applied by gentle rocking for 1h at room temperature. Membranes were then washed vigorously three times in T-TBS, and visualization of binding location and quantity of secondary antibody was achieved via chemiluminescent substrate (ECL-Plus, GE Healthcare). Membranes were then washed gently in T-TBS for five minutes, re-blocked to ensure minimal background noise caused by nonspecific binding of the antibody to the membrane, and detection was repeated as above using monoclonal mouse PCNA antibodies (Cell Signaling Technologies) at a dilution of 1:2000 and HRP-conjugated anti-mouse secondary at a dilution of 1:3000. For Study 1 and Study 3, blots were then reprobed

using the same methodology with polyclonal goat Mcm2 antibodies (Santa Cruz) at a dilution of 1:200 and HRP-conjugated anti-goat secondary at a dilution of 1:10000.

## 2.5 Densitometry and statistical analysis

In order to assess whether Cdc7 and PCNA protein levels differed between groups on a particular blot, ImageJ (Rasband, 1997-2009) was used to assess the mean gray value on a rectangle of constant size encompassing each band in turn. The corresponding Ponceau-stained image was then converted to 8 bit grayscale, and the mean gray value of a different rectangle of constant size encompassing a thin sample of the entirety of each lane was measured. The Ponceau values were used to normalize the bands to correct for possible differences in total protein loading between lanes which might occur due to inaccuracy of the Bradford assay. In the event that a single blot would not accommodate all desired groups, each group was divided evenly between blots. Half of each group appeared on each blot, to evenly distribute between-blot variation across all groups. HeLa cell lysate was used to normalize between blots prior to statistical analysis. All comparisons were done using Excel to perform the Student's t-test and assuming unequal variances.

# Chapter 3. Results

## 3.1 Animal weights

Zucker rats were weighed at the time of termination to compare obese rat weight to that of their lean counterparts at early and late termination times (see Figure 7). Lean and obese rats terminated during the first 24h post-AOM injection (Study 1) weighed approximately 148g and 268g, on average, respectively, while those terminated at 32 wks post-AOM injection (Study 2) weighed approximately 260g and 722g on average, respectively (Figure 9). Obese rats were significantly heavier than their lean littermates both immediately following AOM injection and at the time tumours appeared. However, significantly greater weights for both obese and lean rats at the late timepoint than the early timepoint indicates the rats were still engaged in developmental growth.

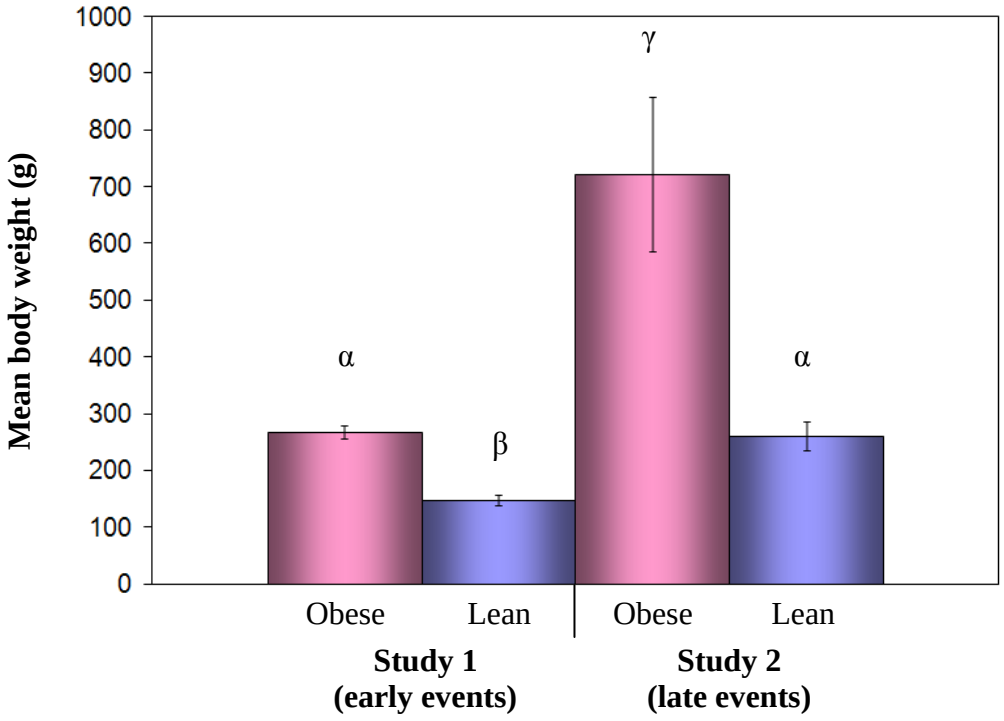



**Figure 9. Obese rats are significantly heavier than age-matched lean rats at both termination timepoints.** Obese 8-week-old rats (early events) were similar in weight to lean 39-week-old rats, highlighting the difference in the phenotype. Values are means ± standard deviation. Bars with different letters (α, β or γ) represent weight values which are significantly different ($p < 0.05$) as determined by z-test.

## 3.2 Study 1: Early events in colon carcinogenesis in Zucker obese and lean rats

### 3.2.1 Cdc7 levels are increased in lean colonic mucosa relative to obese by 3h post-AOM

Since Cdc7 levels are relatively constant throughout the cell cycle (Masai et al., 2005), we reasoned that any difference in Cdc7 protein level between obese and lean colon immediately following DNA damage should be attributable to impairment of the DNA damage response in the obese state. We therefore assessed Cdc7 levels in obese and lean colon 3h, 9h and 24h after carcinogen administration as well as in age-matched uninjected animals (Figure 10-Figure 13).

The Cdc7 antibody consistently generated two bands in both the HeLa positive control and the rat samples: a strong band at approximately 64kDa (consistent with the theoretical respective weights of human and rat Cdc7 which are 64kDa and 62kDa); and a fainter co-migrating band at approximately 84kDa. Previous studies have demonstrated multiple co-migrating bands in human samples using the same antibody, which were unaffected by siRNA depletion of Cdc7, suggesting that bands which do not appear near 64kDa are not the product of antibody binding to Cdc7 (Tenca et al., 2007). Therefore, only the 64kDa bands were quantified.

**Figure 10. No significant differences were seen in Cdc7 or PCNA expression between the colonic mucosa of obese and lean uninjected Zucker rats.** Whole soluble protein extracts from colonic mucosa of unchallenged Zucker obese and lean rats were homogenized in modified RIPA buffer and soluble proteins were extracted by centrifugation (see **Materials and Methods**). Cdc7 and PCNA protein levels were detected by immunoblotting. Densitometric quantitation of resulting bands was normalized to that of total protein (measured by Ponceau S staining) for each sample. Resulting values averaged over total number of samples are shown graphically as means ± standard deviation ($n_{obese}$=5; $n_{lean}$=6). Densitometry was performed using Image J software (NIH).

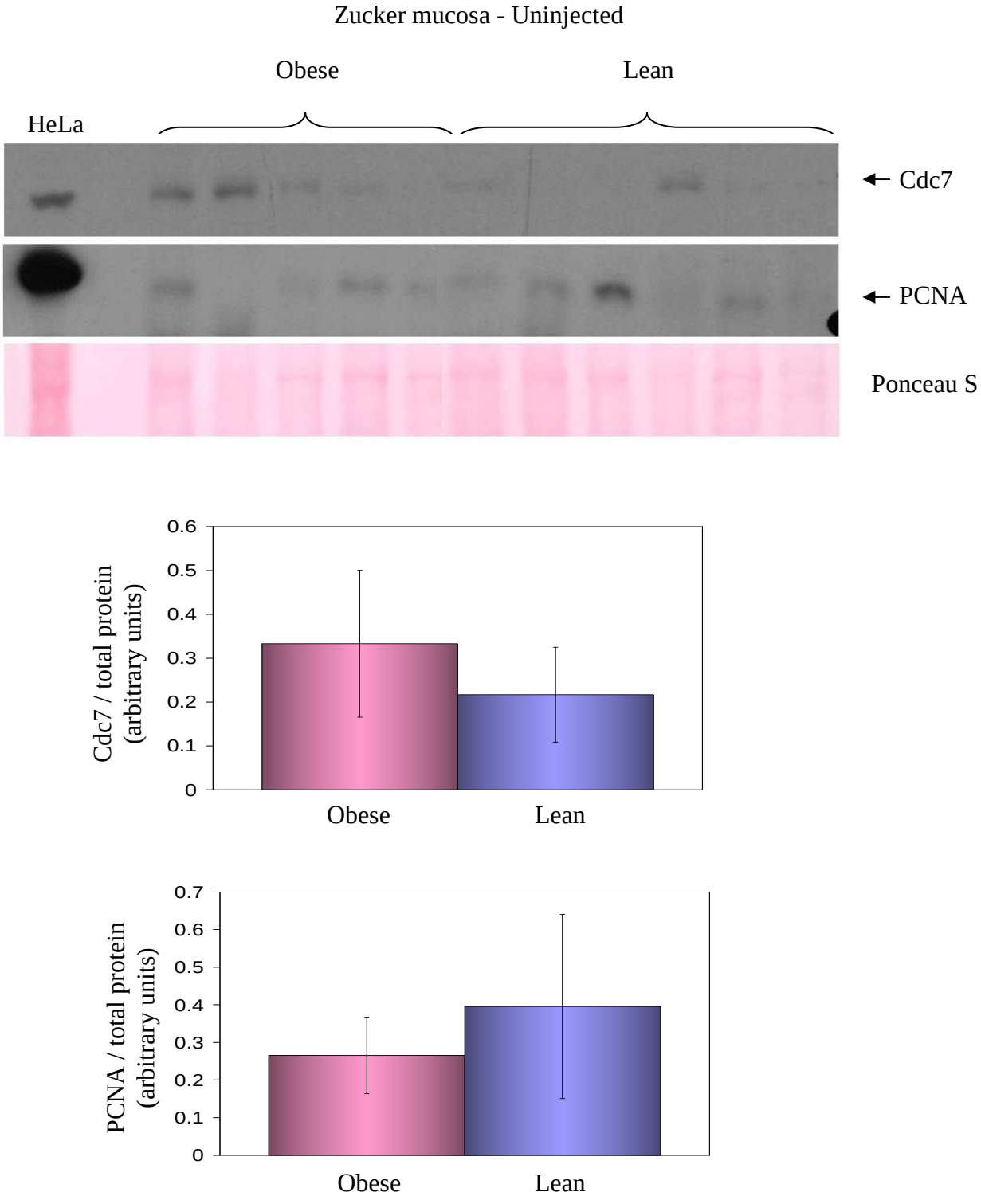

Zucker mucosa - Uninjected
Obese
Lean
HeLa
Cdc7
PCNA
Ponceau S
Cdc7 / total protein (arbitrary units)
0.6
0.5
0.4
0.3
0.2
0.1
0
Obese
Lean
PCNA / total protein (arbitrary units)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Obese
Lean

**Figure 11. Higher Cdc7 levels in the colonic mucosa of lean relative to obese rat colonic mucosa 3h post-carcinogen challenge is consistent with checkpoint-associated upregulation of Cdc7 favoured in lean rats.** Whole soluble protein extracts from mucosa of challenged Zucker obese and lean rats collected 3 hours after AOM challenge were prepared as for uninjected Zucker rats (see Figure 10). Cdc7 and PCNA protein levels were detected by immunoblotting. Densitometric quantitation of resulting bands was normalized to that of total protein (measured by Ponceau S) for each sample. Resulting normalized measurements were averaged over the total number of samples in each group and are shown graphically as means ± standard deviation ($n_{obese}$=6; $n_{lean}$=6). Densitometry was performed using Image J software (NIH). Asterisk (*) indicates significant difference between obese and lean ($p<0.05$). PCNA levels did not differ significantly between obese and lean rats at this timepoint.

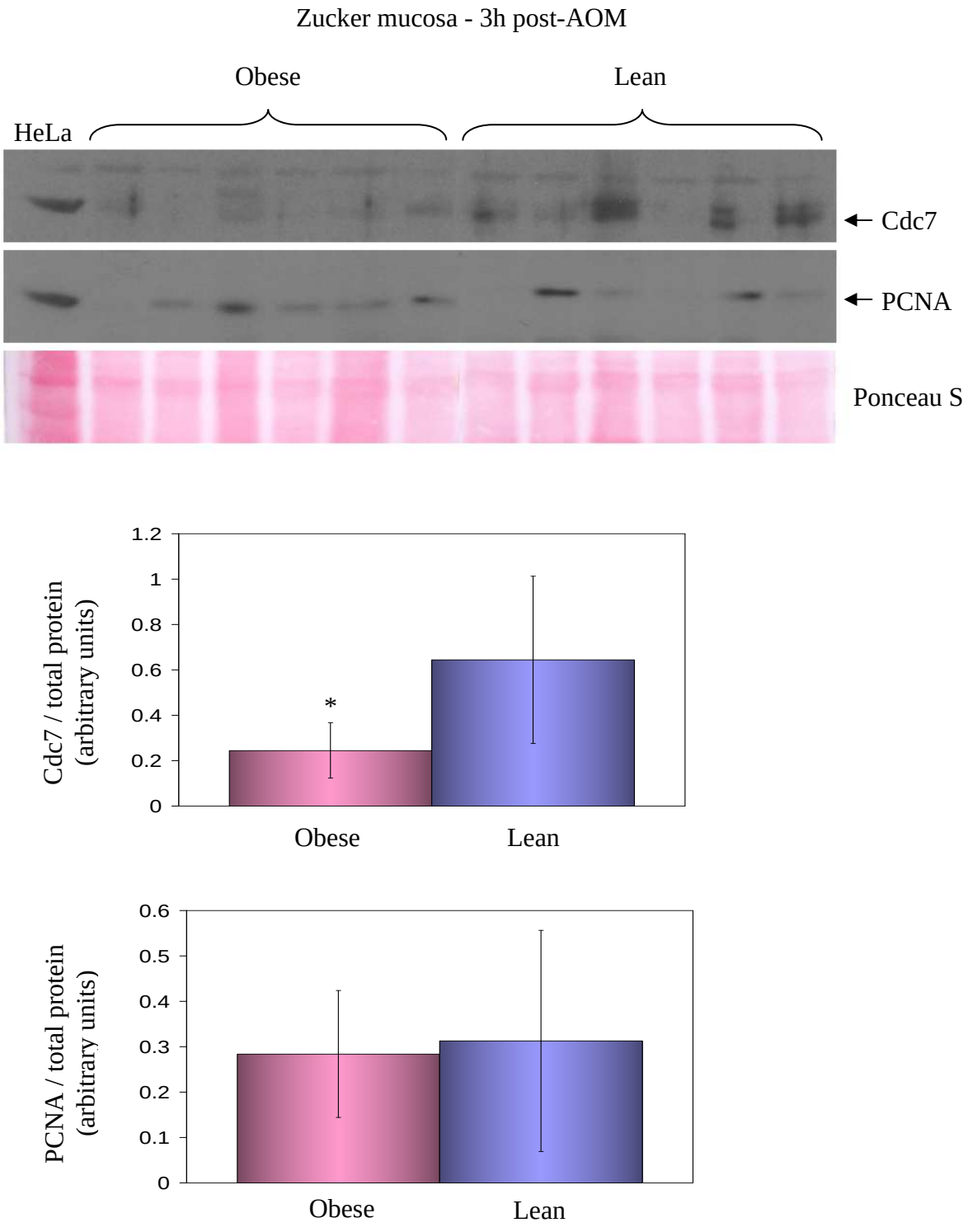
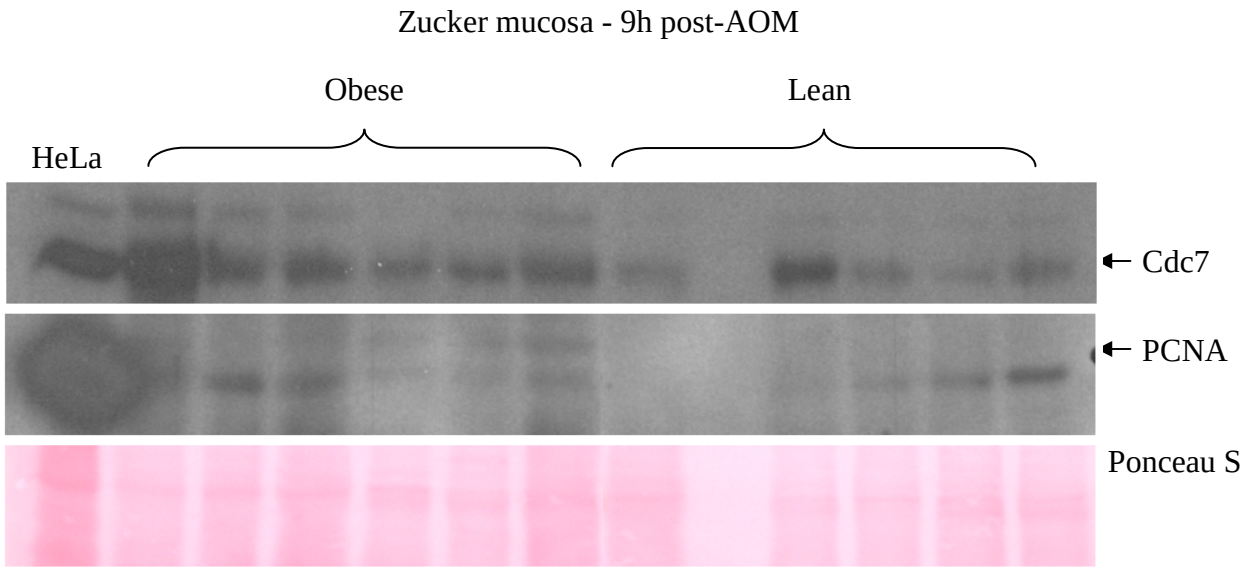
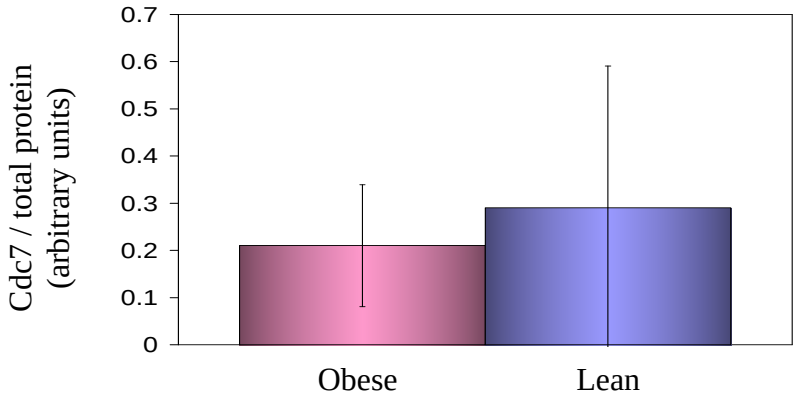

Zucker mucosa - 3h post-AOM
Obese
Lean
HeLa
Cdc7
PCNA
Ponceau S
Cdc7 / total protein (arbitrary units)
1.2
1
0.8
0.6
0.4
0.2
0
*
Obese
Lean
PCNA / total protein (arbitrary units)
0.6
0.5
0.4
0.3
0.2
0.1
0
Obese
Lean

**Figure 12. No significant difference in Cdc7 or PCNA expression was seen in the colonic mucosa of lean relative to obese rats 9h post-carcinogen challenge.** Whole soluble protein extracts from colonic mucosa of challenged Zucker obese and lean rats collected 9 hours after AOM challenge were prepared as for previous Zucker rats (see Figure 10, Figure 11). Cdc7 and PCNA protein levels were detected by immunoblotting, and resulting bands were quantified by densitometry. Resulting measurements were normalized to that of total protein (measured by Ponceau S staining) for each sample and averaged over the total number of samples in each group. Normalized protein levels are shown graphically as means ± standard deviation ($n_{obese}$=6; $n_{lean}$=5). Densitometry was performed using Image J software (NIH).


Zucker mucosa - 9h post-AOM
Obese
Lean
HeLa
Cdc7
PCNA
Ponceau S


Cdc7 / total protein
(arbitrary units)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Obese
Lean

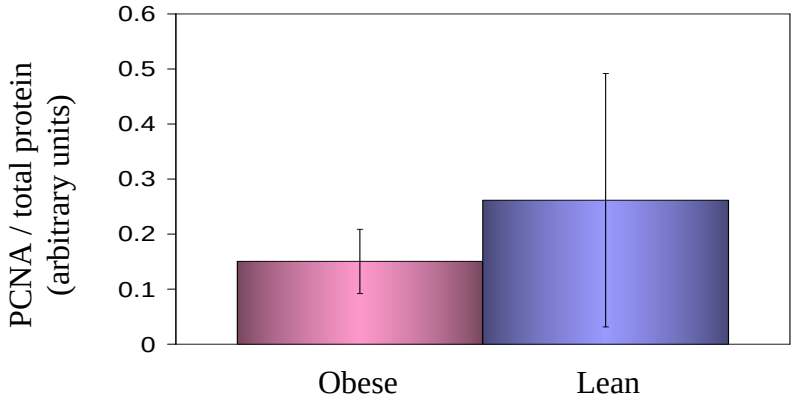
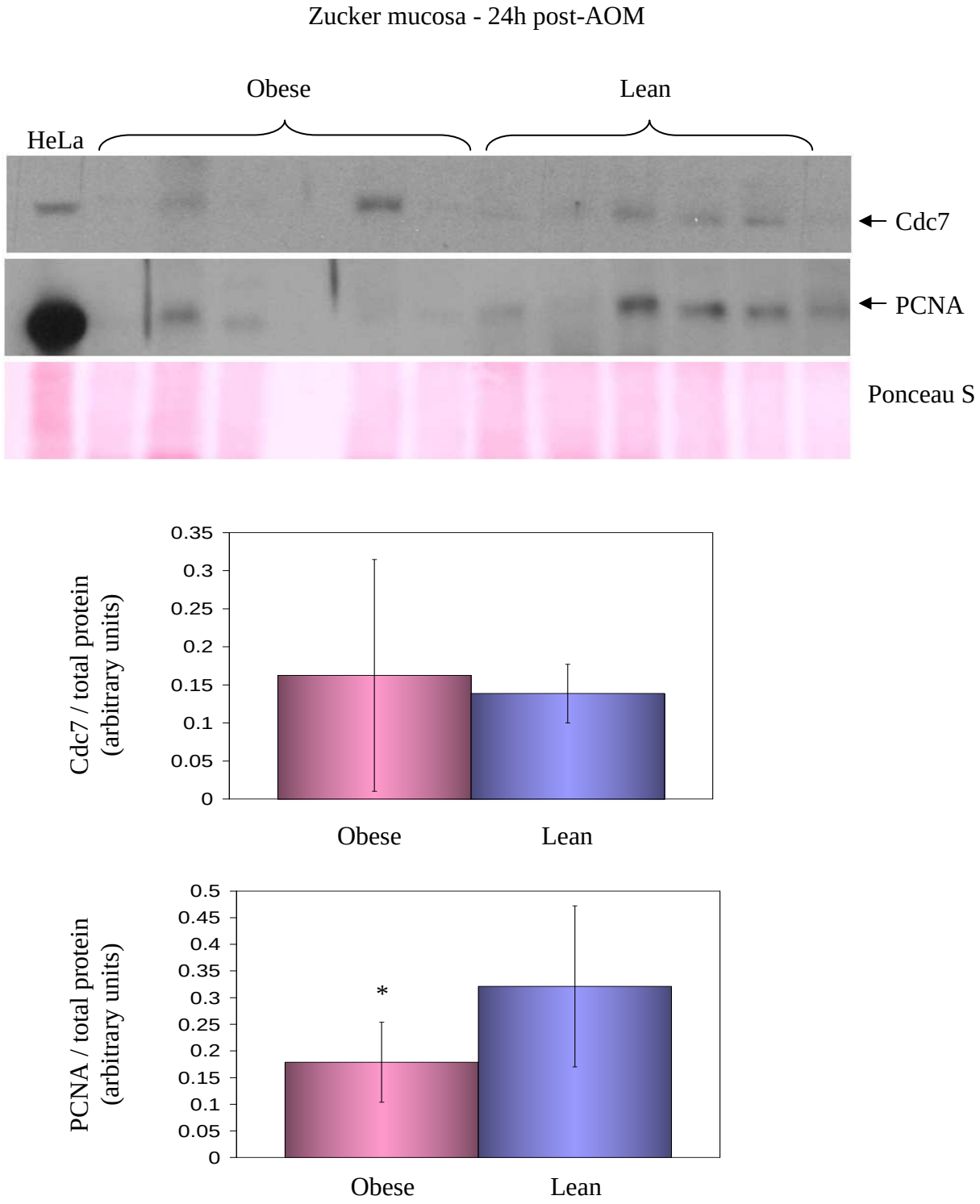

PCNA / total protein
(arbitrary units)
0.6
0.5
0.4
0.3
0.2
0.1
0
Obese
Lean

**Figure 13. Higher PCNA levels in lean relative to obese rat colonic mucosa 24 hours after DNA damage is consistent with PCNA role in DNA damage repair favoured in lean rats.** Whole soluble protein extracts from colonic mucosa of challenged Zucker obese and lean rats collected 24 hours after AOM challenge were prepared as for previous Zucker rats (see Figure 10-Figure 12). Cdc7 and PCNA protein levels were detected by immunoblotting, and resulting bands were quantified by spot densitometry. Measurements obtained were normalized to that of total protein (measured by Ponceau S staining) for each sample and averaged over the total number of samples in each group. Normalized levels are shown graphically as means ± standard deviation ($n_{obese}$=5; $n_{lean}$=6). Densitometry was performed using Image J software (NIH). Asterisk (*) indicates significant difference between obese and lean ($p<0.05$).


Zucker mucosa - 24h post-AOM
Obese
Lean
HeLa
Cdc7
PCNA
Ponceau S
Cdc7 / total protein (arbitrary units)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Obese
Lean
PCNA / total protein (arbitrary units)
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
Obese
Lean

No significant difference between lean and obese mucosal samples in Cdc7 or PCNA levels were seen (Figure 10), although statistical trends were toward increased Cdc7 in obese rat colonic mucosa ($p\sim0.11$) and decreased PCNA in obese rat colonic mucosa ($p\sim0.13$) relative to lean rat colonic mucosa. High variability between subjects from the same group was noted. These animals were female and were not necessarily on a common ovulatory cycle. It is possible that cyclic variation in hormone levels caused natural variability in levels of these proteins. It is noteworthy that no significant upregulation of either Cdc7 or PCNA was seen in colonic mucosa of obese rats relative to lean rats prior to AOM administration. Since tissues of obese animals are known to have higher proliferation rates (Mena et al., 2009), it is reasonable to believe that obese colon would express significantly more cell cycle proteins, including both Cdc7 and PCNA, than lean colon. Possibly, Cdc7 expression differences are not as pronounced in growing rats due to globally enhanced proliferation in tissues of both groups relative to other stages of development. That the two replication factors showed opposing trends suggests that an increase in Cdc7 level in response to proliferation signaling alone is not necessarily accompanied by concomitant upregulation of PCNA. Whether this uncoupling of expression is due to more efficient degradation of PCNA or as a result of Cdc7 functions not involving PCNA is unclear.

In contrast to uninjected animals, Cdc7 levels were increased in lean animals relative to obese as early as 3h post-AOM (Figure 11), suggesting that upregulation of Cdc7 in response to DNA damage occurs at a relatively early timepoint. PCNA levels were not significantly different between lean and obese rat colonic mucosa in uninjected rats.

Cdc7 upregulation at 3h post-AOM was only discernible through comparison of lean and obese animals, as neither the lean nor the obese Cdc7 expression time course showed significant differences between timepoints (see Figure A 1 and Figure A 2 in the Appendix), possibly due to smaller sample size used to create the time courses (n=5-6 vs n=3 per group). Non-significant trends in patterns of expression in these time courses showed a drop in Cdc7 expression in obese rat colonic mucosa ($p\sim0.08$) which was not seen in lean. This suggests that differences in Cdc7 level seen at 3h post-AOM between obese and lean colonic mucosa could be the result of combined inhibition of Cdc7 expression to prevent origin firing during fork stalling in both rat phenotypes and concomitant or immediately subsequent upregulation of Cdc7 to support a more robust checkpoint signaling response in lean animals.

### 3.2.2 PCNA levels are not significantly higher in lean colonic mucosa relative to obese until after 9 hours post-AOM

By 9 hours post-AOM, significant differences between obese and lean rat mucosal levels of Cdc7 had reverted (Figure 12), suggesting that upregulation of Cdc7 in association with checkpoint initiation is transient. Obese and lean rat colonic mucosal PCNA level differences at this timepoint remained statistically non-significant, suggesting that PCNA upregulation is not required for early checkpoint processes.

In contrast to Cdc7, PCNA levels were significantly increased in lean animals relative to obese at the 24 hour timepoint following DNA damage (Figure 13), indicating that PCNA upregulation during the DNA damage response occurs well after the initial rise in Cdc7. This is consistent with Cdc7 involvement in early checkpoint events and increased expression of PCNA following successful DNA damage repair. Similar to results for Cdc7 levels, PCNA

upregulation was detected only when lean rat colonic mucosa was compared to obese, as PCNA levels did not differ significantly between timepoints for either obese or lean (see Figure A 1 and Figure A 2 in the Appendix). Again, the lack of significant differences is possibly due to insufficient sample size in the timecourse experiments (n=5-6 vs. n=3 per group). Non-significant trends in PCNA levels in these timecourse experiments show a less dramatic increase between 9h and 24h post-AOM in obese rat colonic mucosa ($p\sim0.13$) than lean rat colonic mucosa ($p\sim0.10$), supporting impairment of obese DNA damage repair regulatory systems.

## 3.3 Study 2: Late events in colon carcinogenesis in Zucker obese and lean rats

### 3.3.1 Cdc7, but not PCNA, abundance is significantly higher in obese colon mucosa relative to lean late in carcinogenesis

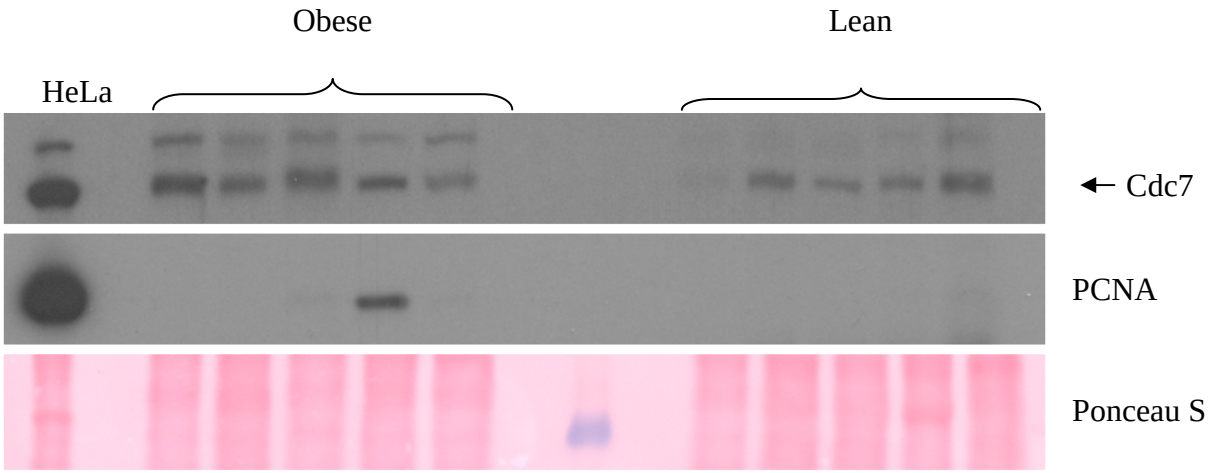
In order to examine the relative abundance of Cdc7 and PCNA in tumours in an obese rat model, the remaining Zucker rats were sacrificed at 32 weeks post-AOM. Unfortunately for statistical purposes, only 1 tumour was collected from the remaining 10 lean Zucker rats. Five obese Zucker rats produced a total of 6 tumours among them, thus clearly demonstrating the increased risk of CRC in obese animals.

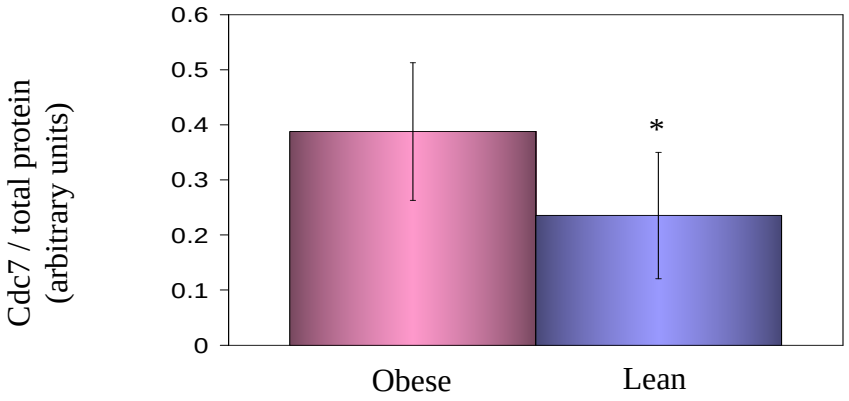
Since obese rats are at higher risk to develop tumours and they have higher numbers of and larger ACF than lean rats late in carcinogenesis (Raju & Bird, 2003), it was hypothesized that obese animals would have increased mucosal hyperplasia relative to lean animals, causing a concomitant upregulation in replication factors such as Cdc7 and PCNA.

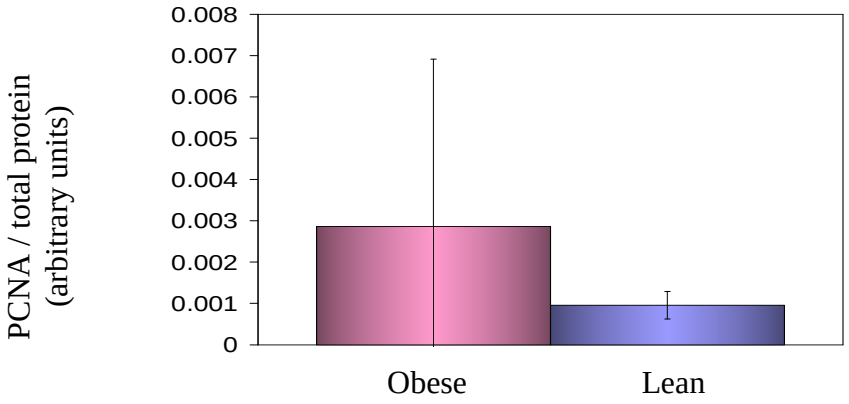
Cdc7 and PCNA levels were assessed by western blot in obese vs. lean Zucker non-tumour mucosa 32 weeks after AOM injection. Results showed that, in contrast to the pattern during early timepoints, obese colon expressed higher amounts of Cdc7 than lean in the late timepoint (Figure 14), consistent with increased cellular proliferation signaling from obese rat adipocyte populations (see section 1.8). PCNA levels, however, were unexpectedly not significantly increased in obese rat colonic mucosa vs. lean (Figure 14). This suggests that upregulation of initiation factors in response to proliferation signaling is not sufficient to increase abundance of all fork components. In particular, PCNA expression may be inhibited post-transcriptionally (Tommasi & Pfeifer, 1999).

**Figure 14. Cdc7 levels are higher in obese normal-appearing colonic mucosa vs. lean once tumours have appeared.** Whole soluble protein extracts from non-tumour mucosa of Zucker obese and lean rats collected 32 weeks following AOM challenge were prepared as for previous Zucker rats (see Figure 10-Figure 13). Cdc7 and PCNA protein levels were detected by immunoblotting, and resulting bands were quantified by spot densitometry. Measurements obtained were normalized to that of total protein (measured by Ponceau S staining) for each sample and averaged over the total number of samples in each group. Normalized levels are shown graphically as means ±standard deviation ($n_{obese}$=5; $n_{lean}$=5). Densitometry was performed using Image J software (NIH). Asterisk (*) indicates significant difference between obese and lean mucosa ($p<0.05$).

Zucker mucosa - 32 wks post-AOM



### 3.3.2 PCNA, but not Cdc7, levels are significantly higher in obese tumours relative to adjacent mucosa

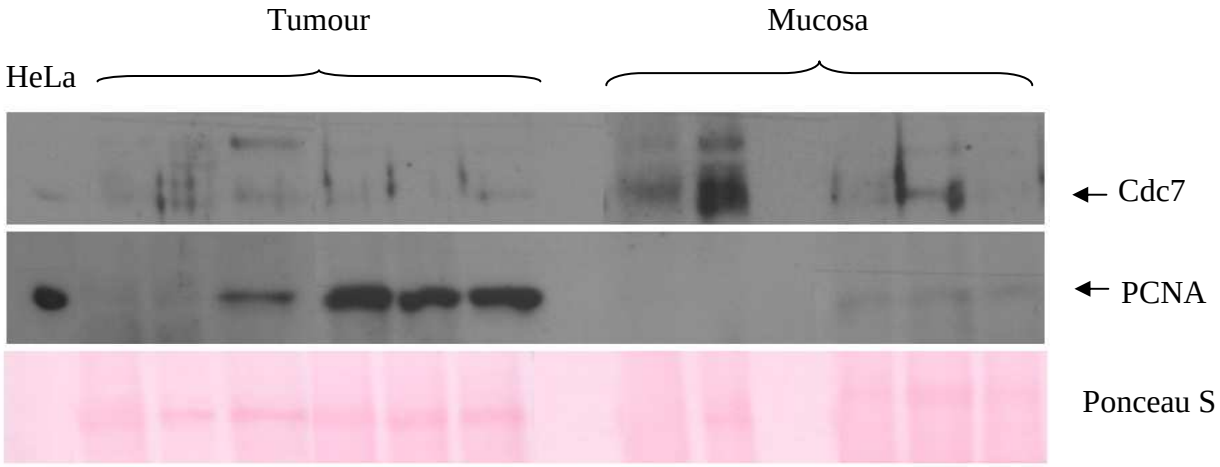
PCNA has been commonly used as a tumour marker for a multitude of tissues from animal models as well as humans (reviewed in Semple & Duncker, 2004). Previous studies have demonstrated that Cdc7 is upregulated in tumours compared to adjacent colon mucosa, as judged by RT-PCR analysis of rat tissues (Hess et al., 1998). More recent studies have shown similar results in human cell lines and resected human samples (Bonte et al., 2008). To assess whether increased Cdc7 and PCNA levels seen in human tumour tissue would extend to an obese rat model, Cdc7 and PCNA expression were compared between tumours and adjacent mucosa from Zucker obese rats terminated 32 weeks after AOM injection. Quantification for Cdc7 was made difficult by an unusual amount of nonspecific binding of unknown origin (Figure 15) between lanes. The resulting background noise persisted on all subsequently developed films, and its etiology remained undiagnosed despite extensive troubleshooting. Nevertheless, inspection of Figure 14 shows mucosal levels of Cdc7 which are approximately equal to or greater than those of obese rat colonic tumours, which supports densitometric measurements showing that obese rat colonic mucosa did not significantly differ from obese tumour tissue in Cdc7 levels at this late timepoint. Combined with results which demonstrate that obese mucosal Cdc7 levels are significantly higher than those of their lean counterparts at this late timepoint (Figure 14), it is likely that abnormal proliferation signaling in obese non-tumour mucosa results in upregulation of mucosal Cdc7 which is not seen in non-obese tissues.

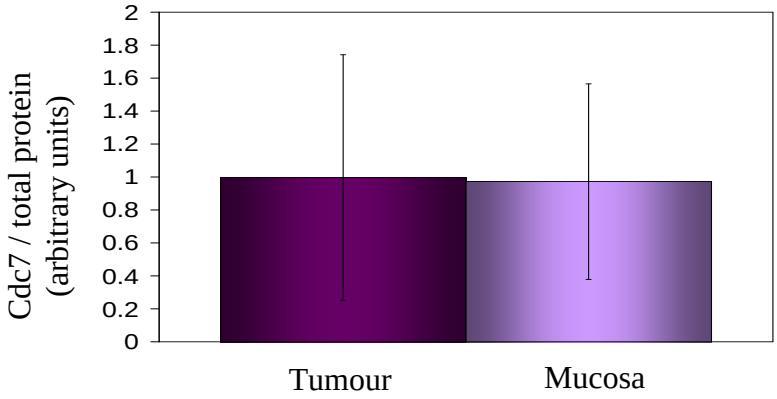
PCNA, but not Cdc7, appeared more abundant in the single lean tumour than adjacent mucosa (see Figure A 3 in the Appendix), supporting PCNA as a more sensitive tumour marker than Cdc7. Both Cdc7 and PCNA expression appeared comparable between some obese tumour samples and the single lean one (see Figure A 4 in the Appendix), suggesting that neither may be a marker of tumour growth aggression.

Results of comparisons of Cdc7 and PCNA levels between obese and lean rat tissues are summarized in Figure 16. Quotient of mean values (obese/lean; denoted OL) was determined to show obese rat tissue protein levels relative to lean. OL greater than 1 indicates timepoints at which obese animals had higher colonic mucosal levels of Cdc7 or PCNA than lean animals. A decreased Cdc7 OL between uninjected animals and those terminated 3h post-AOM (Figure 16) combined with early timelines showing a non-significant decrease in Cdc7 colonic mucosal levels in both obese and lean rats between colonic mucosa over the same period (see Figure A 1 and Figure A 2 in the Appendix) suggests that Cdc7 levels drop in both lean and obese rat colonic mucosa but to a lesser extent in lean. The reduction in Cdc7 levels in lean animals during the first 3h post-AOM suggests that even in a system where the DNA damage response is robust, total Cdc7 is reduced following DNA damage. This overall reduction could result from combined downregulation of Cdc7 to inhibit late origin firing and upregulation of Cdc7 to support checkpoint signaling. Impaired checkpoint signaling in obese rats would then cause them to express less total Cdc7 than lean rats. Following this initial drop, Cdc7 OL levels climb steadily (Figure 16), as do Cdc7 levels of both obese (Figure A 1) and lean (Figure A 2) rat colonic mucosa. Taken together, these results suggest that proliferation signaling is steadily increasing throughout carcinogenesis in both phenotypes,

**Figure 15. Obese colonic mucosa expresses similar levels of Cdc7, but not PCNA, to obese colonic tumours.** Whole soluble protein extracts from colonic tumours and colonic mucosa of Zucker obese rats collected 32 weeks after AOM challenge were prepared as for previous Zucker rats (see Figure 10-Figure 14). Cdc7 and PCNA protein levels were detected by immunoblotting, and resulting bands were quantified by spot densitometry. Measurements obtained were normalized to that of total protein (measured by Ponceau S staining) for each sample and averaged over the total number of samples in each group. Normalized levels are shown graphically as means ± standard deviation ($n_{tumours}$=6; $n_{mucosa}$=5). Densitometry was performed using Image J software (NIH). Asterisk (*) indicates significant difference between obese tumour and mucosa ($p<0.05$).

Zucker obese – 32 wks post-AOM



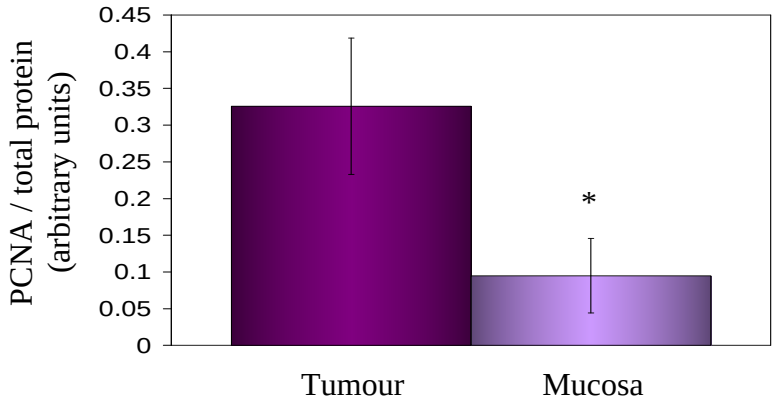
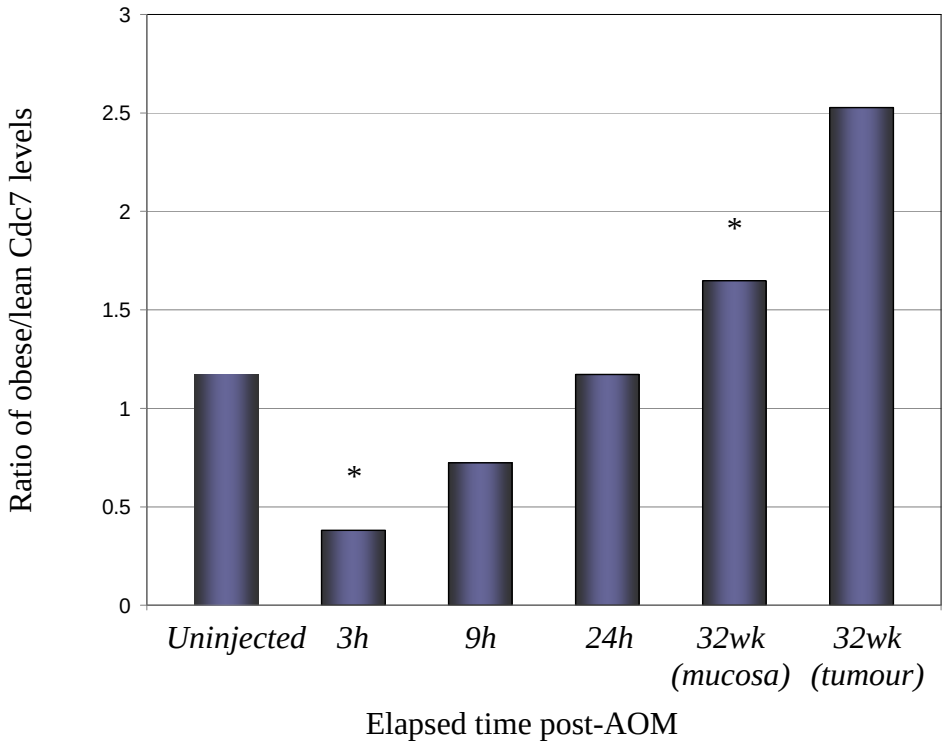
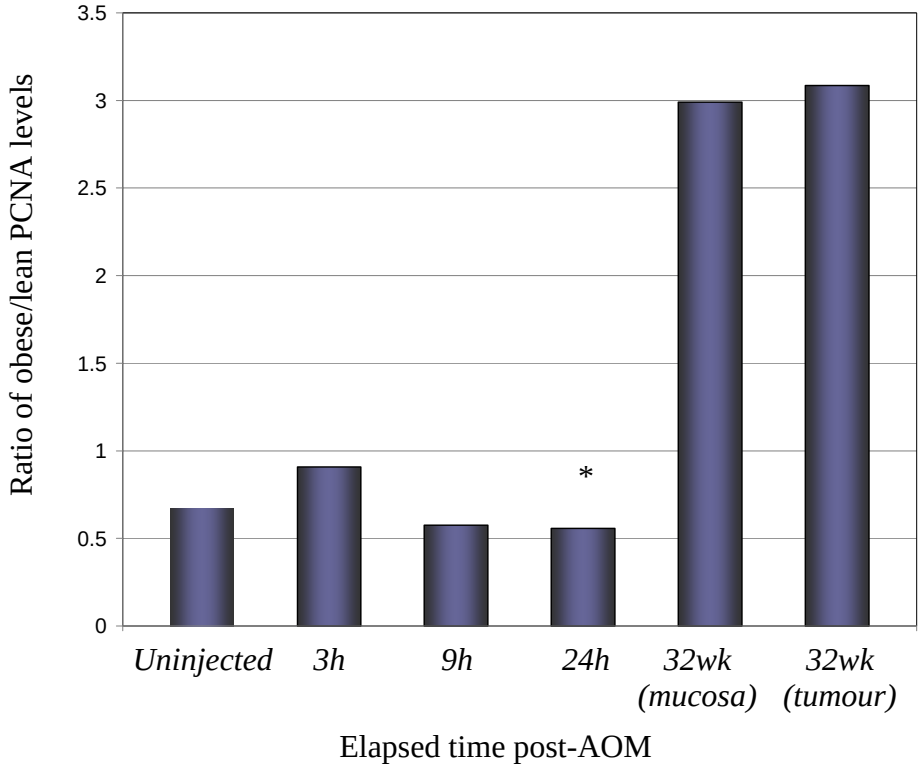



**Figure 16. Summary of obese vs. lean rat colonic protein expression during colon carcinogenesis: Ratio of obese to lean rat colonic Cdc7 levels correlates with expected degree of hyperproliferation, whereas increased ratio of obese to lean rat colonic PCNA levels appears to indicate presence of neoplasia. A.** Ratio of obese to lean rat colonic mucosal Cdc7 levels initially drops, suggesting successful late origin inhibition is coupled with impaired checkpoint response in obese animals. Cdc7 levels then increase in obese relative to lean rat colonic mucosa with time. **B.** In contrast to relative levels of Cdc7, obese rat colonic mucosal PCNA levels are similar to that of lean rats (approximately 1:1) at least during early carcinogenesis. An increase in the ratio of obese to lean PCNA levels in 32-week-post-AOM mucosa results from high variability in obese rat colonic mucosal PCNA levels at that timepoint, which were not significantly different from values for lean rats (see Figure 14). There was only a single lean tumour, so it was not possible to determine if obese rat colonic tumours differed significantly in protein expression from lean rat colonic tumours. Asterisk (*) indicates significant difference ($p<0.05$) in original comparison of obese to lean rat colonic mucosal levels.

A.


Ratio of obese/lean Cdc7 levels
3
2.5
2
1.5
1
0.5
0
*
*
Uninjected
3h
9h
24h
32wk (mucosa)
32wk (tumour)
Elapsed time post-AOM

B.


Ratio of obese/lean PCNA levels
3.5
3
2.5
2
1.5
1
0.5
0
*
Uninjected
3h
9h
24h
32wk (mucosa)
32wk (tumour)
Elapsed time post-AOM

but to a greater extent in obese animals. PCNA OLs, in contrast, are uniformly close to 1 during early timepoints, suggesting that PCNA levels are not affected by obese metabolic state the same way Cdc7 levels are, possibly due to post-transcriptional inhibition of PCNA. At 32 weeks post-AOM, an apparent increase in PCNA OL levels is seen to result from a single obese rat colonic mucosal sample (Figure 14), suggesting that PCNA OL levels remain constant until a threshold population of dysplastic or neoplastic cells is present. This population is expected to be larger in obese animals at this late timepoint because they have enhanced tumour risk.

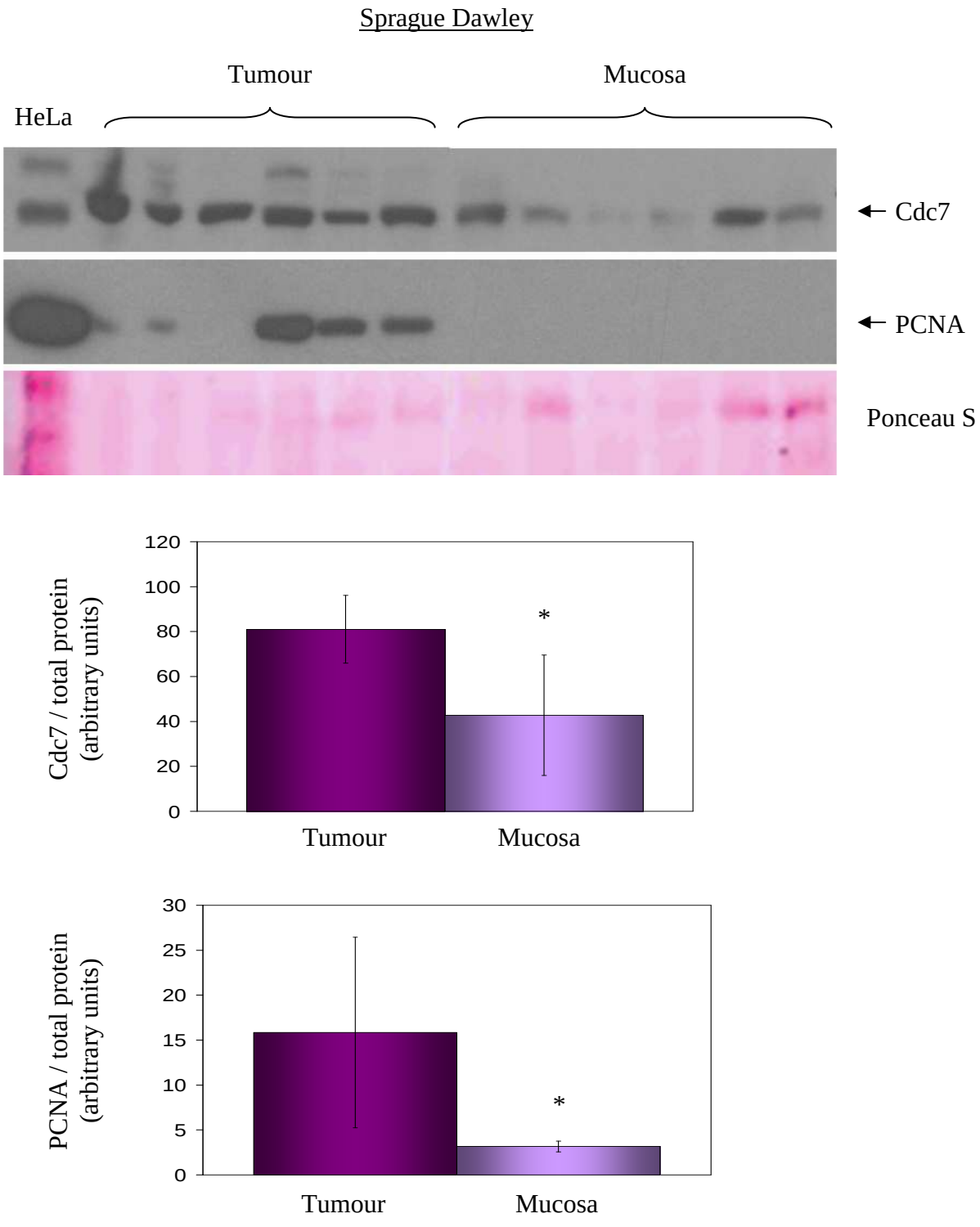
Tumour tissue is not the same as non-tumour tissue, and hence it is not always obvious whether protein factor expression should be increased or decreased in colonic tumours depending on their metabolic environment. Both Cdc7 and PCNA OL levels were increased compared to early timepoints (Figure 16). However, because only a single lean tumour sample was available, statistical determination of differences in Cdc7 and PCNA levels between obese and lean rat colonic tumours was not possible. Inspection of Figure A 4 nevertheless shows that the majority of obese tumour samples expressed more Cdc7 and PCNA than the single lean tumour sample. Whether obese rat colonic tumours have higher levels of these replication factors, consistent with enhanced proliferation signaling in obese tumours, or obese and lean rat colonic tumours have expression profiles more closely resembling each other than the normal tissue from which they arose, is unclear from these results.

## 3.4 Study 3: Late events in colon carcinogenesis in the Sprague Dawley rat model

### 3.4.1 Cdc7 and PCNA levels are increased in AOM-induced colonic tumours in Sprague Dawley rats

Animal research in colon cancer is not restricted to obese rat models. The utility of a protein biomarker as a research tool relies in part on its extendability to species which are in common use. To confirm that Cdc7 and PCNA protein levels are upregulated in common laboratory rat tumour tissue, we compared Cdc7 and PCNA expression in AOM-induced tumour tissue to adjacent mucosal tissue from Sprague Dawley rats via western blot. Results showed increased Cdc7 and PCNA levels in tumour tissue relative to adjacent mucosa, ($p<0.05$; Figure 17), consistent with previous studies in human cell lines and human tissues (Bonte et al., 2008) and compatible with dysregulation of cellular proliferation in tumour tissue. Significantly higher Cdc7 levels in Sprague Dawley tumours than mucosa contrasts with results showing no difference between tumour and mucosal Cdc7 levels in Zucker obese animals (Study 2), suggesting that the utility of Cdc7 as a biomarker may rely on metabolic state. This dependence is not shared by PCNA, suggesting it may be a more robust tumour marker in general. However, Cdc7 expression was increased in all six of the Sprague Dawley tumour samples, but only four of the six tumour samples expressed more PCNA, suggesting that Cdc7, like many other pre-replication factors, is a more sensitive tumour marker. Overall, these results support the utility of both Cdc7 and PCNA as colonic tumour markers in the Sprague Dawley rat.

**Figure 17. Cdc7 and PCNA levels are significantly increased in tumours vs. normal-appearing colon mucosa in Sprague Dawley rats.** Whole soluble protein extracts from tumours and remaining mucosa of Sprague Dawley rats 32 weeks post-AOM challenge were prepared by homogenization in modified RIPA buffer followed by centrifugation. Extracted proteins were resolved by SDS-PAGE and immunoblotted with antibodies for Cdc7 and PCNA. Densitometric quantitation of resulting bands was normalized to that of total protein (measured by Ponceau) for each sample. Resulting values averaged over total number of samples are shown graphically as means ± standard deviation ($n_{tumours}$=6; $n_{mucosa}$=6). Densitometry was performed using Image J software (NIH). Asterisk (*) indicates a significant difference between tumour and mucosa ($p<0.05$).


Sprague Dawley
Tumour
Mucosa
HeLa
Cdc7
PCNA
Ponceau S
Cdc7 / total protein (arbitrary units)
120
100
80
60
40
20
0
*
Tumour
Mucosa
PCNA / total protein (arbitrary units)
30
25
20
15
10
5
0
*
Tumour
Mucosa

As previously mentioned, mucosa adjacent to rat colonic tumours is hyperproliferative, typically with ACF numbers of over 200 and average crypt multiplicity of more than 4 (Shivapurkar et al., 1996). Interestingly, PCNA was barely detectable in mucosa relative to tumours, while Cdc7 showed a strong signal in mucosa, with tumour levels stronger still (Figure 17). These results suggest that Cdc7 may additionally be a more sensitive marker of hyperproliferation than PCNA.

Since Mcm2 is a target of Cdc7 kinase activity and has previously been shown to be upregulated in dysplastic colonic crypts (Scott et al., 2003), we attempted to evaluate the samples for MCM2 levels using antibodies which were purported to detect rat antigen; however, the antibody, which clearly binds the HeLa (human) positive control antigen, does not appear to bind at all to the Sprague Dawley rat samples (see Figure A 5 in the Appendix). To assess whether the lack of affinity of the Mcm2 antibody for Sprague Dawley antigen is unique to that strain, we used it to assess mucosal samples from Zucker lean rats, another strain of *Rattus norvegicus* (see Figure A 6 in Appendix). Results were similar to those for Sprague Dawley samples, supporting a low primary antibody affinity for rat antigen.

# Chapter 4. Discussion

To summarize the results of this investigation, western blot analysis was used to assess the protein expression of Cdc7 and PCNA during colon carcinogenesis in AOM-induced obese and lean rat models. It was shown that during the DNA damage response, Cdc7 levels were elevated in lean animals relative to obese animals well in advance of a similar pattern for PCNA levels (see Figure 11 and Figure 13), consistent with an early role for Cdc7 in the checkpoint response and a later role for PCNA in repair. Additionally, we showed that Cdc7 and PCNA levels are elevated in colonic tumours relative to adjacent mucosa in the non-obese Sprague Dawley rat model, but Cdc7 levels do not differ between obese tumours and mucosa.

We propose that Cdc7 is upregulated in lean animals as part of the ATR-Chk1 checkpoint response immediately upon fork stalling due to the presence of bulky alkyl DNA adducts induced by AOM. The increase in Cdc7 abundance is delayed or impaired in obese animals, due to cytokine- and fatty acid-mediated impaired DNA damage response (Mena et al., 2009; Zeng et al., 2008). Obese animals are consequently at increased risk of sustaining irreversible DNA damage following fork collapse and therefore accumulate more transformed cells than lean animals. Combined with increased baseline cellular proliferation rates in untransformed obese mucosal tissues due to obesity-associated hyperinsulinemia (Mena et al., 2009), this heightened risk of DNA damage could contribute to the greatly augmented tumour incidence seen in obese rats. In colonic tumour tissue, PCNA levels are also increased relative to normal-appearing colonic mucosa in both Sprague-Dawley and obese Zucker rats, suggesting that excessive upregulation of fork complex factors is an event tolerated only by

neoplastic cells. PCNA upregulation may be accomplished in tumour cells by inhibition of normally-occurring post-transcriptional repression (Tommasi & Pfeifer, 1999).

It is possible that the resistance of tumours to damage caused by a highly increased replication rate is balanced by increases in expression of DNA damage response factors, including Cdc7 and PCNA. The checkpoint factors ATM, Chk2 and p53 are all upregulated in breast cancer (Gochhait et al., 2009), although it is not known whether this upregulation extends to colonic tumours. Normal cells require less Cdc7 than tumour cells for viability, as Cdc7 inhibitors selectively kill tumour cells (Menichincheri et al., 2009; Montagnoli et al., 2008). Tumour cells may therefore be more heavily reliant on DNA damage response factors such as Cdc7 and PCNA during early events. Thus, the involvement of both Cdc7 and PCNA in DNA replication and DNA damage response appears to enhance their utility as tumour markers.

## 4.1 DNA damage response appears more robust in lean than obese

Obese Zucker rats have previously been shown to develop more and larger ACF than their lean counterparts in response to carcinogen administration, despite having the same levels of DNA damage and BrdU-labeled cells per crypt (Koch et al., 2008). In the current study, it was found that lean Zucker rats express more Cdc7 protein than obese Zucker rats soon after AOM injection, at a time when the DNA damage response cascade is first being initiated. The reported role of Cdc7 in claspin phosphorylation during the ATR/Chk1 checkpoint response (Kim et al., 2008) suggests that upregulation of Cdc7 could play a part in modulating the efficacy of Chk1 activation. This leads to the possibility that the increased

Cdc7 levels in lean relative to obese colonic mucosa following AOM injection results from the lean rats having mounted a more robust checkpoint response than the obese rats, consistent with an increased rate of ACF and tumours in obese animals.

## 4.2 Temporal order of Cdc7 and PCNA upregulation is consistent with respective roles in DNA damage signaling and fork restart

Previous work has suggested that the Cdc7-mediated ATR checkpoint lies upstream of DNA damage repair processes in which PCNA is involved (Kim et al., 2008; Kastan & Bartek, 2004; Moldovan et al., 2007). Following the ATR checkpoint, ubiquitin-modified PCNA is involved in some DNA damage repair pathways (reviewed in Moldovan et al., 2007). It is unclear whether modified PCNA moieties can be deubiquitylated or must be replaced with newly expressed PCNA following successful repair (Moldovan et al., 2007) causing a concomitant increase in PCNA levels. Monoubiquitylation of PCNA, induced by the presence of some DNA lesions, leads to recruitment of alternative polymerases whose conformation accommodates the lesion (Lee & Myung, 2008). Polyubiquitylation of PCNA is thought to be associated with error-free template switching, though the mechanism of this repair pathway is poorly understood (Lee & Myung, 2008; Moldovan et al., 2007). In the event of template switching, one proposed mechanism is that polyubiquitylated PCNA is detached from polymerase δ or ε and marks the site of the lesion; the replicative polymerase then traverses the lesion without the original tether, and a new PCNA molecule must replace the original one to restore polymerase processivity and replisome cohesion (Moldovan et al., 2007). By comparing the DNA damage response in lean rats to the impaired pathway seen in

obese rats, this study showed that Cdc7 upregulation (Figure 11) precedes PCNA upregulation (Figure 13), consistent with these models.

Cdc7 activity is required to maintain viability during S-phase checkpoint in multiple eukaryotic species (Kim et al., 2008; Ogi et al., 2008; Tenca et al., 2007; Tsuji et al., 2008). The cellular response to fork stalling involves Cdc7-mediated claspin mediation of Chk1 activation (Kim et al., 2008). Even within the same organism, some authors argue that the resulting checkpoint signal inactivates Cdc7 (Costanzo et al., 2003; Heffernan et al., 2007). It is not clear from the present data whether these differences result from a relative increase in protein levels in lean animals due to a more robust checkpoint response or a relative decrease in protein levels in obese animals due to Cdc7 downregulation supporting late origin inhibition. However, time course data showed a trend toward an initial drop in Cdc7 levels between obese uninjected control mucosa and that of AOM-challenged obese animals 3 hours after injection (Figure A 1) which was not seen in lean animals (Figure A 2). Both obese phenotypes subsequently showed a slow rising trend during the following timepoints, suggesting that the difference in Cdc7 levels 3h post-AOM was due to a relative decrease in obese protein levels. While it would be simple to conclude that obese checkpoint more efficiently downregulates Cdc7 following DNA damage, that would be inconsistent with increased risk for tumour development in obese animals. It is more likely that multiple pathways cause Cdc7 to be effectively downregulated to prevent late origin firing in both phenotypes but more effectively upregulated to support checkpoint signaling in lean rats.

## 4.3 Cdc7 protein upregulation in tumours does not extend to the obese rat model

This study demonstrated that the differences in Cdc7 expression between tumour and mucosa seen in human tissues and cell lines (Bonte et al., 2008) do not extend to the obese rat model. Hyperinsulinemia characteristic of obese animals (Argiles, 1989) is known to increase cell proliferation signaling (Mena et al., 2009). Cdc7 levels may be upregulated in response to these aberrant signals.

Hyperinsulinemia does not appear to cause an increase in PCNA levels to the extent that it increases Cdc7 levels in obese animals relative to lean animals in non-tumour colonic mucosa at early timepoints (Figure 16). Early timepoints comprise a period during which dysplastic and neoplastic cells are unlikely to be present, whereas mucosa adjacent to tumour tissue contains dysplastic cells and may contain microscopic neoplasms detectable only on histological examination. In this study, tumour samples originated from visible tumours, and mucosal samples originated from the remaining mucosa, which could easily have contained undetected neoplastic cells. Additionally, normal-appearing mucosa from obese rats at late timepoints likely contains more dysplastic and neoplastic cells than that from lean rats at the same timepoint, since obese animals are at higher risk for cellular transformation (Weber et al., 2000). Therefore, the lack of a pronounced increase in PCNA levels comparable to that of Cdc7 levels during early timepoints in obese rat colonic mucosa relative to lean rat colonic mucosa may reflect a low rate of dysplasia and neoplasia in these tissues. Significantly higher PCNA levels in obese rat colonic tumours than non-tumour colonic mucosa (Figure 15) combined with the absence of significant differences between PCNA levels in obese and lean

rat non-tumour colonic mucosa at the same timepoint (Figure 14) suggests that an increase in mucosal PCNA concomitant to that of Cdc7 is not necessarily a consequence of obese cell cycle dysregulation. The lack of correlation between Cdc7 levels and PCNA levels could be due to dissimilar transcriptional promotion, post-transcriptional expression modulation, post-translational degradation, or a combination of these. The PCNA gene encodes an antisense RNA transcribed from a promoter in intron 1, which is thought to mediate post-transcriptional PCNA expression inhibition (Tommasi & Pfeifer, 1999). Transcription of this antisense RNA is activated in part by E2F, which upregulates the Cdc7 activator, ASK (Yamada et al., 2002) and has putative binding sites in the murine Cdc7 promoter (Kim et al., 1998), suggesting that PCNA upregulation follows Cdc7 activity during the normal cell cycle. Gene-specific, PCNA expression inhibition may therefore be accomplished post-transcriptionally through binding of transcription factors which upregulate ASK and may upregulate Cdc7 itself.

Obese mucosa at late timepoints is likely to contain more precancerous tissue than lean, since obese animals are at much higher risk of developing tumours than lean animals. These tissues contain dysplastic cells which may already require an enhanced DNA damage response to maintain viability in the face of dysregulated proliferation, causing Cdc7 levels to necessarily increase to feed the chain of activation. This suggests that obese tumours are more checkpoint competent than obese untransformed tissue, and would therefore likely more closely resemble lean tumours in their expression of checkpoint proteins. While our data was insufficient to statistically test this hypothesis, abundance of both Cdc7 and PCNA in the single lean colonic tumour sample was certainly within the range of that of obese colonic

tumours, supporting the possibility that DNA damage response robustness may be similar between lean and obese tumours.

In the lean rat model, we saw significantly fewer tumours than in the obese rats (1 tumour/10 lean rats vs. 6 tumours/5 obese rats). Previous studies employing a single dose of the AOM precursor 1,2-dimethylhydrazine (DMH) showed that the non-obese Sprague Dawley rat fails to develop macroscopic lesions as late as 41 weeks post-challenge, even at a dose of 125 mg/kg (McLellan et al., 1991b), well above currently employed dosages of 20-30 mg/kg for 1-3 doses (Yusup et al., 2009; Zhi et al., 2007). This is consistent with reduced carcinogen sensitivity in non-obese rats leading to lowered tumour risk.

When challenged with two doses of AOM at 10 mg/kg, obese Zucker rats develop significantly more intermediate (4-6 crypts) and advanced (>7 crypts) ACF, but not significantly more primal ACF (1-3 crypts) or total ACF than lean Zucker rats nine weeks after the second injection (Raju & Bird, 2003), suggesting that obese aberrant crypts are simply more prone to clonal expansion. Also, non-obese Sprague Dawley rats develop *more* total, small and large ACF following two weekly AOM doses at 15 mg/kg body weight than obese Zuckers develop following two weekly doses at 10 mg/kg body weight (Eskin et al., 2007), suggesting that the dose reduction for Zucker is sufficient to slow the early phases of preneoplasia. Our results, however, demonstrate that even with two larger doses of AOM, non-obese Sprague Dawley colonic mucosa does not contain the increased levels of Cdc7 resulting from cellular proliferation enhancement in obese mucosa seen with a single reduced dose.

## 4.4 Cdc7 and PCNA protein upregulation in human tumours extends to a Sprague Dawley rat model

Previous studies examining Cdc7 protein as a marker for colon cancer focused on human samples and cell lines (Bonte et al., 2008). This study demonstrated that Cdc7 protein levels were upregulated in Sprague Dawley rat tumours compared with adjacent mucosa (Figure 17). It is interesting that Cdc7 showed a much stronger signal in the mucosa than PCNA, since mucosa adjacent to tumours is hyperproliferative, containing hundreds of ACF (Shivapurkar et al., 1996). This could be due to the kinetics of upregulation of Cdc7 vs. PCNA in the DNA damage response, DNA replication, or both. Perhaps, for instance, much more Cdc7 is expressed in response to DNA damage than PCNA. This is particularly likely since only a small proportion of lesions which induce the ATR-Chk1 pathway will result in fork collapse and hence PCNA modifications which direct DNA repair mechanisms (Ampatzidou et al., 2006). The observed differences could also result from post-transcriptional inhibition of PCNA expression (Tommasi & Pfeifer, 1999), as described in section 4.3.

## 4.5 Conclusions and future directions

The major findings of this work are:

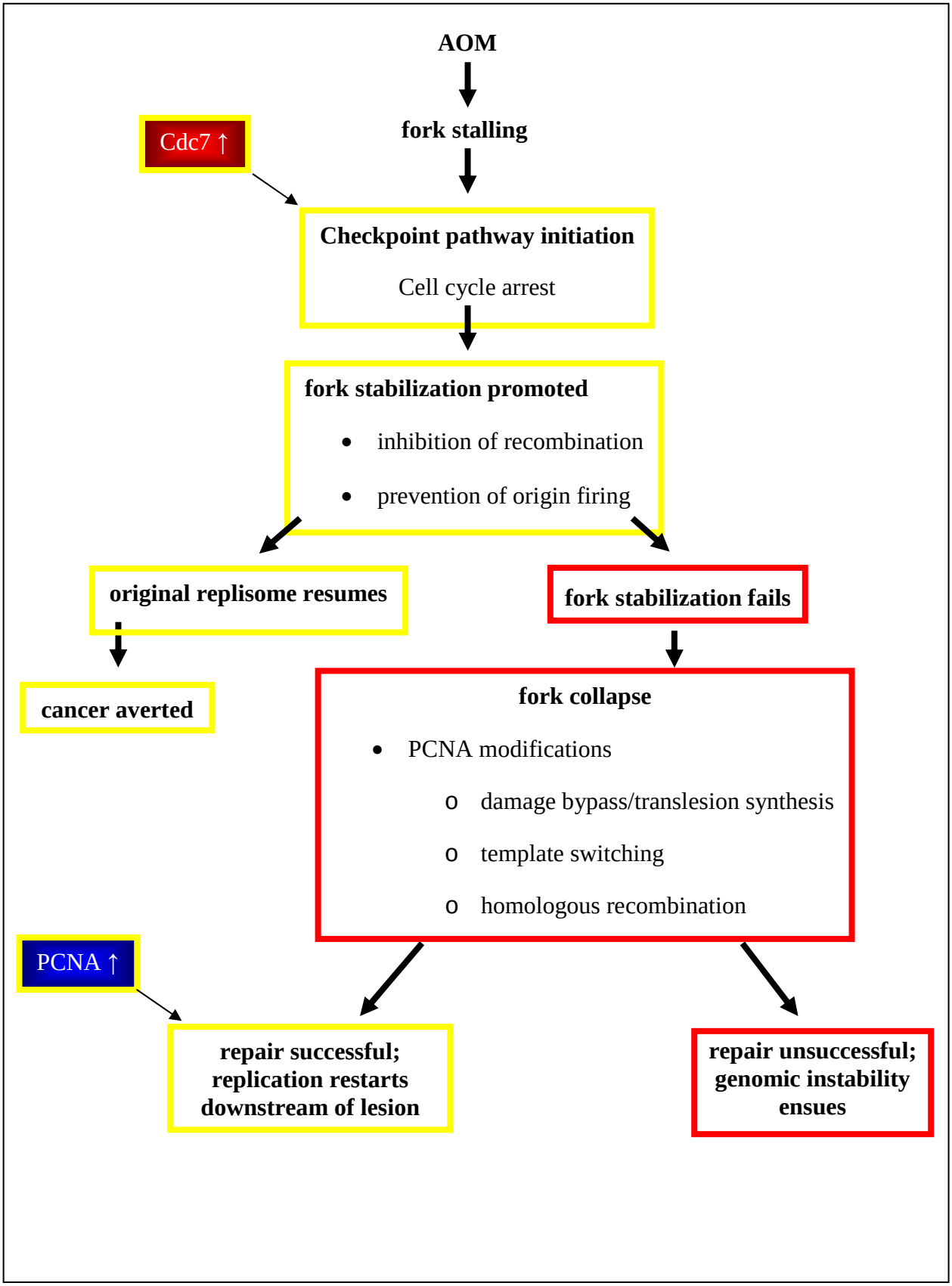
1. The temporal order of Cdc7 and PCNA upregulation immediately following DNA damage induction is consistent with a model in which Cdc7 mediates the early checkpoint response to DNA alkylation-stalled replication forks prior to PCNA upregulation following translesion synthesis;

2. Both Cdc7 and PCNA appear to be elevated in colonic tumours; however, obese metabolic state appears to obscure the difference in Cdc7 level between tumours and colonic mucosa;

3. PCNA remains an effective tumour marker regardless of metabolic state.

A model of the DNA damage response which is supported by the expression patterns seen in this work and which is consistent with previous literature (Branzei & Foiani, 2005; Moldovan et al., 2007) is shown in Figure 18. In lean animals immediately following carcinogen challenge, fork stalling caused by the presence of DNA alkyl adducts has initiated the ATR checkpoint cascade, requiring the upregulation of Cdc7 to mediate claspin phosphorylation necessary for Chk1 activation (Kim et al., 2008). By 24h post-challenge, PCNA is upregulated to replace ubiquitin-modified moieties still encircling the lesion site (Moldovan et al., 2007). In obese animals, neither Cdc7 nor PCNA is upregulated,

**Figure 18. Role of Cdc7 and PCNA in DNA damage response and obese cancer risk.** During early timepoints in lean animals, Cdc7 is upregulated to support ATR-Chk1 pathway initiation by fork stalling. The ATR-Chk1 pathway acts to promote fork stabilization. PCNA is upregulated only in the relatively rare event that fork stabilization is unsuccessful, to replace modified moieties which no longer support DNA polymerase δ/ε processivity. Obese animals suffer from an impaired DNA damage response, which may extend to repair processes. This increases their risk of cellular transformation by reducing the incidence of successful DNA damage repair. Proliferation of obese cells is enhanced by hyperinsulinemia, causing an increased tumour growth rate. Yellow boxes indicate intact DNA damage response pathways and factors which are inhibited in obese animals. Red boxes indicate pathways favoured in obese animals.


AOM
fork stalling
Cdc7 ↑
Checkpoint pathway initiation
Cell cycle arrest
fork stabilization promoted
inhibition of recombination
prevention of origin firing
original replisome resumes
fork stabilization fails
cancer averted
fork collapse
PCNA modifications
damage bypass/translesion synthesis
template switching
homologous recombination
PCNA ↑
repair successful; replication restarts downstream of lesion
repair unsuccessful; genomic instability ensues

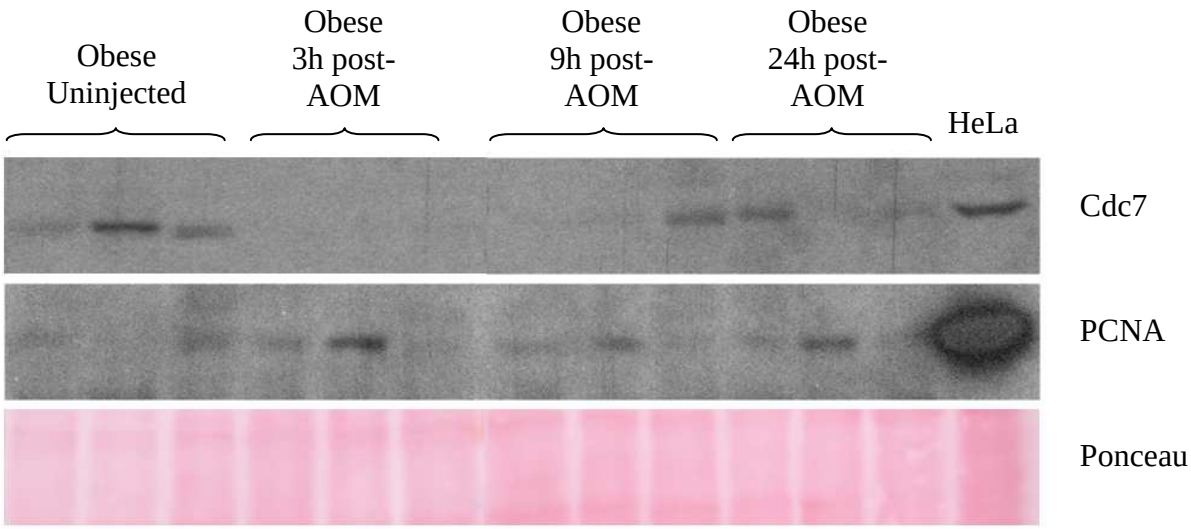
suggesting that pathways leading to successful repair are inhibited. Therefore, there is a pathway shift in obese animals towards unsuccessful DNA damage response and hence towards cancer.

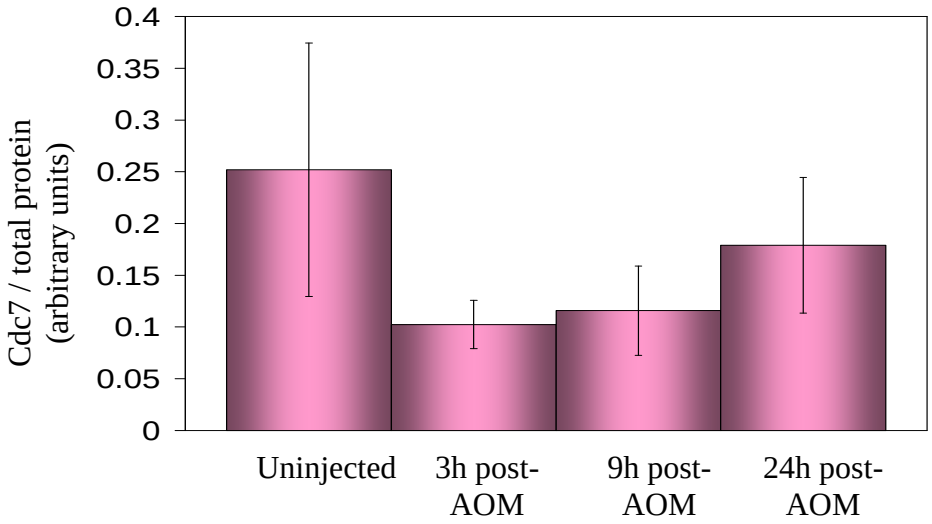
Late in carcinogenesis, colonic mucosa of both obese and lean rats is highly hyperproliferative and likely contains some dysplastic cells (Bird, 1995). At the same time, obese mucosal tissue displays increased Cdc7 expression due to increased cellular proliferation signals in its hyperinsulinemic environment (Mena et al., 2009). Increased Cdc7 expression in obese mucosa therefore eliminates the difference between tumour and mucosal Cdc7 expression seen in the non-obese Sprague Dawley rats.

Our findings have clearly demonstrated that use of a single tumour marker is inadvisable. We have shown that Cdc7 is a more sensitive marker than PCNA but is reliable as a tumour marker only in lean animals, suggesting the need for supplementary markers. Future studies could broadly target the importance of cell cycle factors and factors involved in DNA damage and repair in lean and obese metabolic environments during different stages of colon carcinogenesis using techniques such as gene and protein microarrays. Incorporation of phospho-specific antibodies in the protein microarrays could aid in assessing the activation state of checkpoint signaling factors such as ATR, Chk1 and claspin. Further investigation of these aspects will lead to a better understanding of the DNA damage response and the mechanisms influencing increased cancer risk in obesity.

# Appendix - Supplementary Figures

**Figure A 1. No significant differences in Cdc7 or PCNA level were seen between timepoints during early events in obese Zucker colonic mucosa.** Obese samples used for obese vs. lean comparisons (see Figure 10-Figure 13) were re-blotted to examine differences between timepoints. Densitometry was performed using ImageJ, as before. Group means ± standard deviation are shown graphically. Significant differences as determined by Student's t-test of neither Cdc7 nor PCNA levels between mucosa from obese uninjected (n=3), obese 3h post-AOM (n=3), obese 9h post=AOM (n=3), and obese 24h post-AOM (n=3) were seen.


Obese
Uninjected
Obese
3h post-
AOM
Obese
9h post-
AOM
Obese
24h post-
AOM
HeLa
Cdc7
PCNA
Ponceau


Cdc7 / total protein
(arbitrary units)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Uninjected
3h post-
AOM
9h post-
AOM
24h post-
AOM

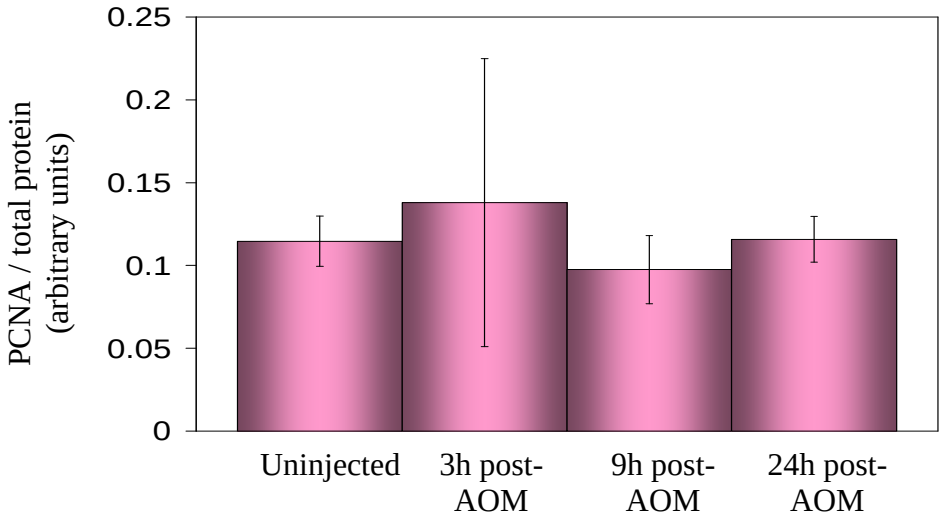
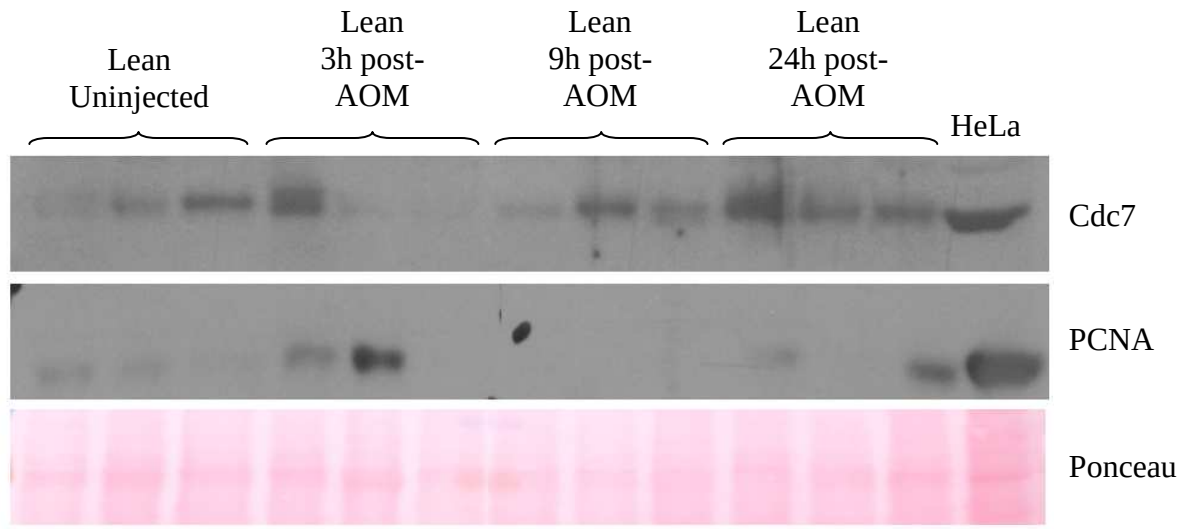
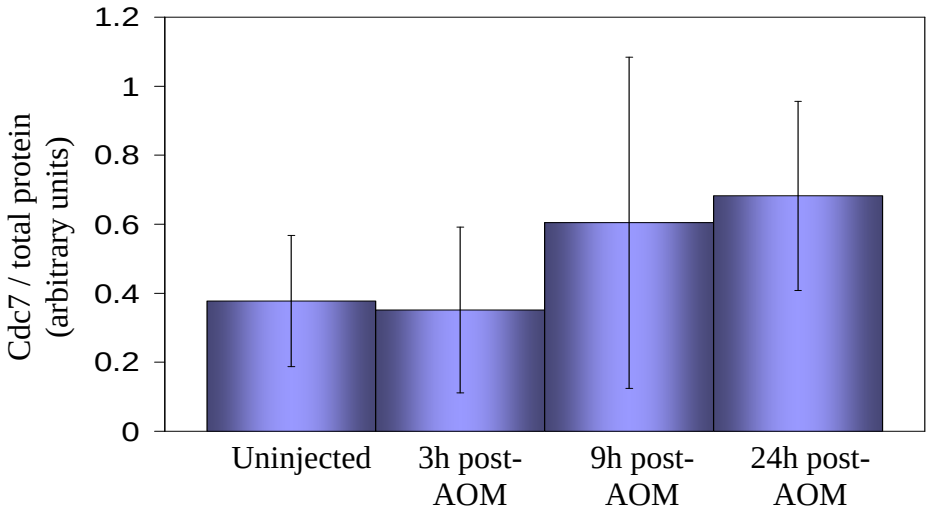
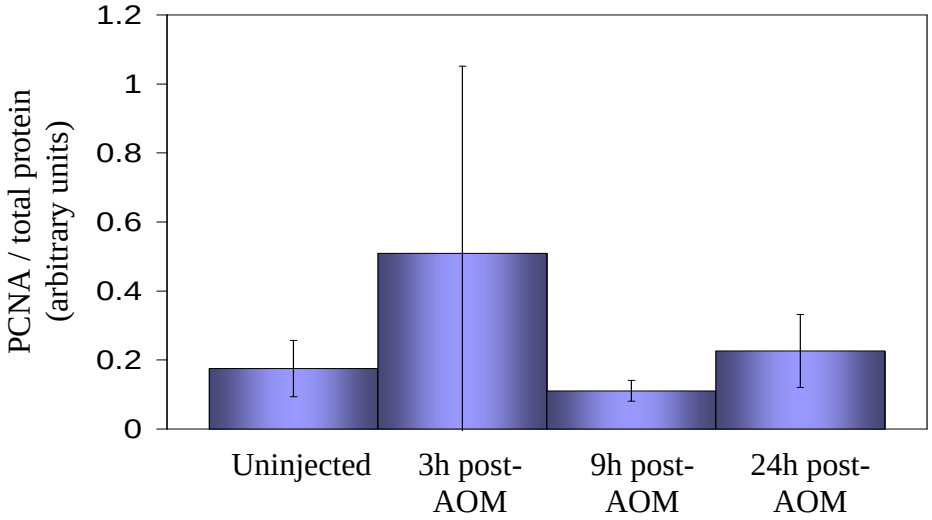

PCNA / total protein
(arbitrary units)
0.25
0.2
0.15
0.1
0.05
0
Uninjected
3h post-
AOM
9h post-
AOM
24h post-
AOM

**Figure A 2. No significant differences in Cdc7 or PCNA level were seen between timepoints during early events in lean Zucker colonic mucosa.** Lean samples used for obese vs. lean comparisons (see Figure 10-Figure 13) were evaluated by western blot to examine differences between timepoints. Densitometry was performed using ImageJ, as before. Group means ± standard deviation are shown graphically. Neither Cdc7 nor PCNA levels differed significantly between colonic mucosa from lean rats which were uninjected (n=3), 3h post-AOM (n=3), 9h post=AOM (n=3), and 24h post-AOM (n=3), as determined by Student's t-test.


Lean
Uninjected
Lean
3h post-
AOM
Lean
9h post-
AOM
Lean
24h post-
AOM
HeLa
Cdc7
PCNA
Ponceau


Cdc7 / total protein
(arbitrary units)
1.2
1
0.8
0.6
0.4
0.2
0
Uninjected
3h post-
AOM
9h post-
AOM
24h post-
AOM


PCNA / total protein
(arbitrary units)
1.2
1
0.8
0.6
0.4
0.2
0
Uninjected
3h post-
AOM
9h post-
AOM
24h post-
AOM

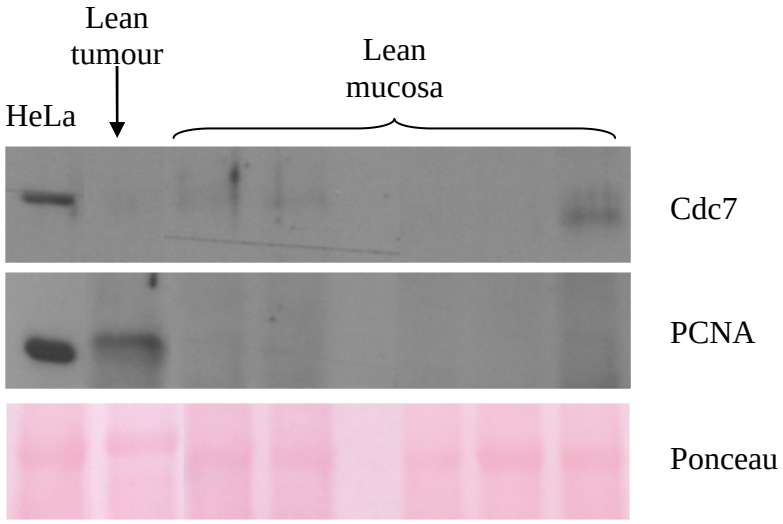



**Figure A 3. The single lean tumour appeared to express more PCNA but not Cdc7 than lean mucosa.** Whole soluble protein extracts from the single colonic tumour (n=1) and colonic mucosa (n=5) of challenged Zucker lean rats collected 32 weeks after AOM challenge were prepared as for previous Zucker tissues (see Figure 10-Figure 15). Cdc7 and PCNA protein levels were detected by immunoblotting.

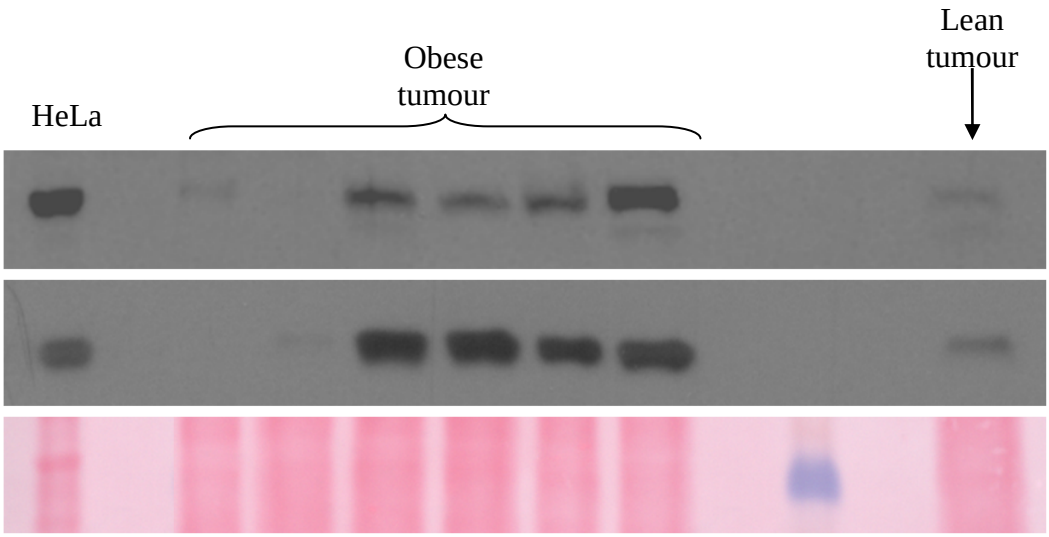



**Figure A 4. The single lean tumour falls within the distribution of obese Cdc7 and PCNA expression.** Obese (n=6) and lean (n=1) colonic tumour samples used for comparison with colonic mucosa were re-blotted for comparison with each other. While the uniqueness of the lean tumour sample made statistical comparisons impossible, visual inspection of the bands shows that the lean tumour sample expresses amounts of both Cdc7 and PCNA which fall in the range of those of the obese tumour samples.

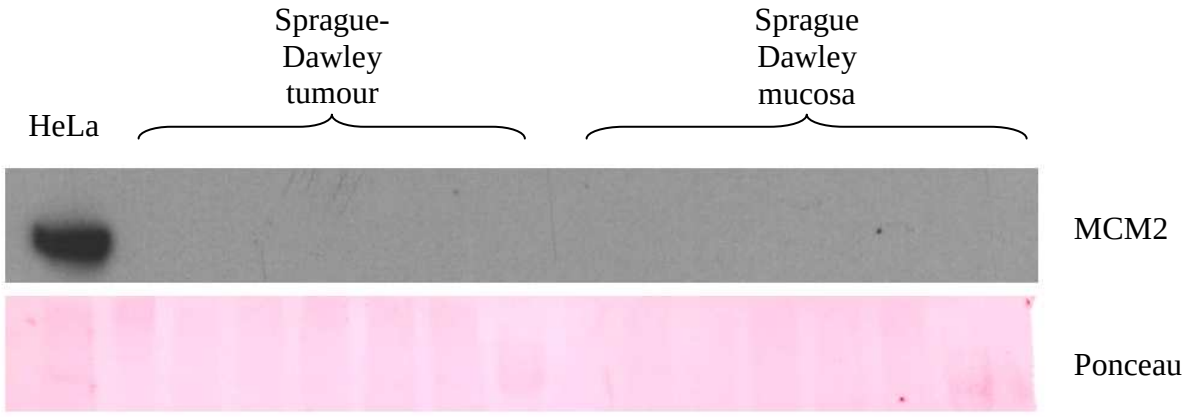



**Figure A 5. MCM2 antibody did not have sufficient affinity for Sprague Dawley protein.** The Sprague Dawley colonic tumour (n=6) and colonic mucosa (n=6) samples shown in Figure 17 were assessed for MCM2 levels using a commercial antibody. The blot was exposed until the signal elicited by the HeLa positive control was saturated. MCM2 signal was undetectable in Sprague Dawley samples.

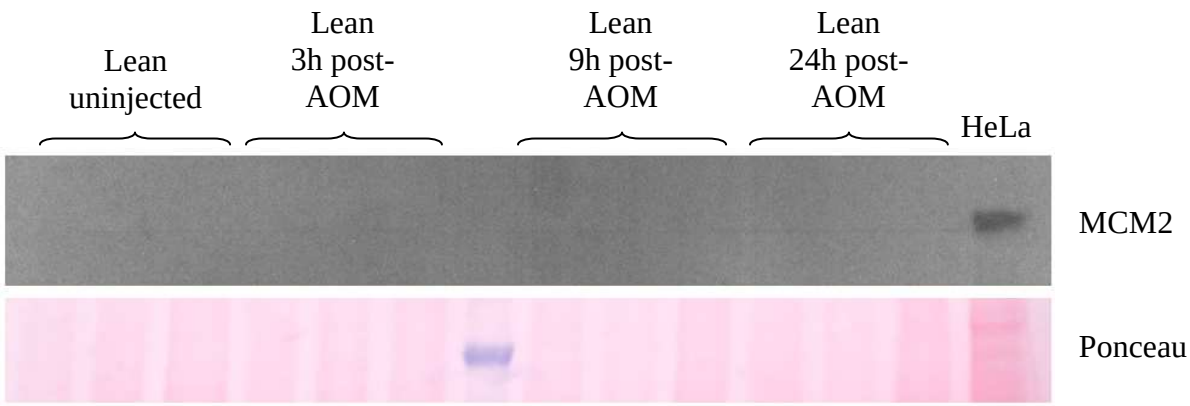



**Figure A 6. MCM2 antibody had insufficient affinity for Zucker antigen.** The blot shown in Figure A 2 was also probed for MCM2 using the same commercial antibody with which the Sprague Dawley samples were probed (see Figure A 5). For this probe, the blot was exposed only until the HeLa showed a strong, visible, non-saturated signal, at which point none of the lean Zucker samples (uninjected, n=3; 3h post-AOM, n=3; 9h post-AOM, n=3; 24h post-AOM, n=3) displayed a visible band.

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