

The role of the chromatin protein HMGN3 in cytokine
induced gene expression

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Abstract

HMGN proteins modulate chromatin structure by binding to chromatin, unfolding nucleosome arrays and altering the pattern of histone modifications. HMGN3 is unique amongst the HMGN family as two splice variants are produced from the HMGN3 transcript. HMGN3 was shown to regulate TNF α -induced expression of the murine chemokine gene CCL1 which is implicated in conditions such as asthma, atherosclerosis and cancer. However, the mechanism by which HMGN3 modulates gene expression was unknown. This investigation studied the role of HMGN3 in modulating TNF α -induced expression of the mCCL1 gene, and identified changes in histone modifications associated with the mCCL1 gene during expression.

Quantitative real-time PCR (QRT-PCR) results show that over-expression of HMGN3 in MEFs reduces mCCL1 induction by TNF α . Chromatin immunoprecipitation (ChIP) assays show HMGN3 binds to the mCCL1 gene. Peaks in Histone H3 lysine 14 acetylation (H3K14ac) coincide with peaks of HMGN3 binding at the mCCL1 gene. TNF α stimulation leads to decreased HMGN3 binding and this correlates with reduced H3K14ac at the mCCL1 gene. This is reminiscent of a previous study that showed HMGN1 inhibits anisomycin-stimulated fosB transcription. Taken together, this shows that HMGN3 fine-tunes TNF α -induced gene expression, and suggests it may be necessary to remove HMGN3 from the mCCL1 gene in order to get efficient transcription.

mCCL1 is known to contain an inducible NF- κ B site. TNF α induces the NF- κ B signalling cascade. TNF α stimulation leads to the translocation of NF- κ B to the nucleus, as well as the kinase IKK α . IKK α can directly phosphorylate H3S10 and enhance acetylation of H3K14 at NF- κ B regulated promoters. p38 also induces phosphorylation of H3S10, which may improve access for NF- κ B to bind the NF- κ B site.

To investigate whether HMGN3 has an indirect role in regulating gene expression, NF- κ B and p38 luciferase assays were performed. Results show that HMGN3 does not significantly affect either the NF- κ B or p38 signalling pathways.

Based on this work and other published data, the following model is proposed: HMGN3 binds mCCL1 chromatin, enhances acetylation of H3K14 as shown by ChIP, and may inhibit TNF α -induced H3S10 phosphorylation, possibly mediated by IKK α , thus inhibiting mCCL1 expression as shown by RNA data. However, following TNF α stimulation, HMGN3 may become phosphorylated, possibly by IKK α or MSK1, reducing HMGN3 binding to chromatin as suggested by the ChIP results, enhancing the accessibility of H3S10 phosphorylation, possibly by IKK α . The reduced binding of HMGN3 may be necessary for efficient expression of the mCCL1 gene.

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Author's Declaration

I, Craig Davis, hereby declare that this thesis is my own work, and to the best of my knowledge, contains no material previously published by another person unless otherwise stated.

Abbreviations

ac	acetylation
Acetyl Co-A	acetyl-coenzyme-A cofactor
ATL	Adult T-cell leukaemia
ATP	adenosine triphosphate
BALF	bronchoalveolar fluid
CAT	chloramphenicol acetyltransferase gene
CBP	CREB-binding protein
CCL1, I-309, TCA3	chemokine (C-C motif) ligand 1
CCR8	chemokine (C-C motif) receptor 8
ChIP	chromatin immunoprecipitation
CHUD	chromatin unfolding domain
cM	centimorgan
CTL	cytolytic T lymphocytes
Da	Dalton
°C	degrees Celsius
Dox	doxycycline
DMEM	Dulbecco's modified eagle's medium
EDTA	ethylenediaminetetraacetic acid
FADD	Fas-associated death domain
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
FRAP	fluorescence recovery after photobleaching
Fusion TA	fusion <i>trans</i> -activator
GAPDH	Glyceraldehyde-3- phosphate dehydrogenase
Glyt1	glycine transporter 1
HAT	histone acetyl transferase
HDAC	histone deacetylase
hr	hour
HL-60	human myeloid leukaemia cell line
HMGN	high mobility group N
HP1	heterochromatin protein 1
IE	immediate early
IκB	inhibitor of κB
IKK	IκB kinase

IKK α	I κ B kinase- α
IKK β	I κ B kinase- β
IKK γ	I κ B kinase- γ
IL-4	interlukin-4
IP	immunoprecipitate
k	kilo
kb	kilobase
Lp(a)	lipoprotein(a)
MDA-MB-435	human breast carcinoma cell line
MEF	mouse embryonic fibroblast
ml	millilitre
MSK1	mitogen- and stress- activated protein kinase
NBD	nucleosome-binding domain
NF-Y	nuclear factor-Y
NF- κ B	nuclear factor- κ B
μ	micro
m	milli
M	molar
n	nano
NKT	natural killer T cells
NLS	nuclear localisation signal
NRE	negative regulatory element
PCAF/GCN5	p300/CBP-associated factor
PE	pseudomonas exotoxin domain III
ph	phosphorylation
PKC	protein kinase C
PITX2	paired-like homeodomain transcription factor 2
QRT-PCR	quantitative real time polymerase chain reaction
RIP	receptor-interacting proteins
RLU	relative light units
RNAi	RNA interference
Rpd3p	reduced potassium dependency-3 protein
RPM	revolutions per minute
RSC	remodel the structure of chromatin
RSK2	ribosomal S6 kinase
RT	room temperature

SEM	standard error of the mean
SD	standard deviation
SODD	silencer of death domain
SWI/SNF	switch/sucrose nonfermenting
TAF	TATA-binding protein-associated factor
TE	tris, EDTA
TBE	tris, borate, EDTA
TBP	TATA binding protein
TCR	T cell receptor
T _H 1	T helper 1
T _H 2	T helper 2
TNF α	tumour necrosis factor alpha
TNF-R1	TNF α - receptor 1
TRADD	TNF receptor-associated death domain
TRAF2	TNF-R-associated factor 2
T _{REG}	T regulatory cells
TSS	transcription start site
VKC	vernal keratoconjunctivitis
Wnt	wingless and int
x g	times gravity

1 Introduction

Eukaryotic cells contain DNA in complex with protein in the nucleus. This complex, called chromatin, serves to compact and protect the DNA molecule and to control access of the cellular machinery to the genetic information. Compaction occurs on various levels (Figure 1) (Felsenfeld *et al.*, 2003). Organisation begins with 146 base pairs of DNA wrapped around eight histone proteins, forming a complex called the nucleosome, an 11 nm structure (Luger *et al.*, 1997). Nucleosomal arrays fold to form two stack 30 nm chromatin fibres where short-range internucleosomal interactions occur (Dorigo *et al.*, 2004). These fibres further compact into higher-order structures, however, the structural details remain to be determined. These extended chromatin fibres form long-range fibre-fibre interactions that ultimately result in a highly organised and condensed chromosome as observed during mitosis with a light microscope.

The hierarchy of condensed DNA must also permit access of cellular machinery to carry out essential processes such as transcription, replication and repair. The nucleosome lies at the heart of the condensed chromatin fibre and plays an important role in regulating access by facilitating reorganisation of chromatin. The nucleosome core particle consists of four proteins called histones: H2A, H2B, H3 and H4. Two H3-H4 dimers form a central tetramer to which two dimers of H2A-H2B interact with to form a histone octomer, the nucleosome (Luger *et al.*, 1997). The histone N-terminal tails protrude from the nucleosome and are susceptible to trypsinolysis (Bohm and Crane-Robinson, 1984), hence only certain tail regions are visible in the X-ray studies by Luger *et al.* The visible regions of the N-terminal tail of Histone H3 and H2B extend from the core particle and pass through the DNA minor groove. Tails participate in forming the 30nm fibre and are important targets for post-translational modifications including acetylation, methylation and phosphorylation (Quina *et al.*, 2006). These histone modifications induce structural changes of the chromatin architecture by disrupting intra- and internucleosomal interactions and thus alter the accessibility of the cellular machinery to the DNA. These modifications also provide recognition and binding sites for a host of transcription factors, proteins and other histone modifying enzymes. Importantly, these epigenetic modifications change the chromatin architecture but do not alter the underlying

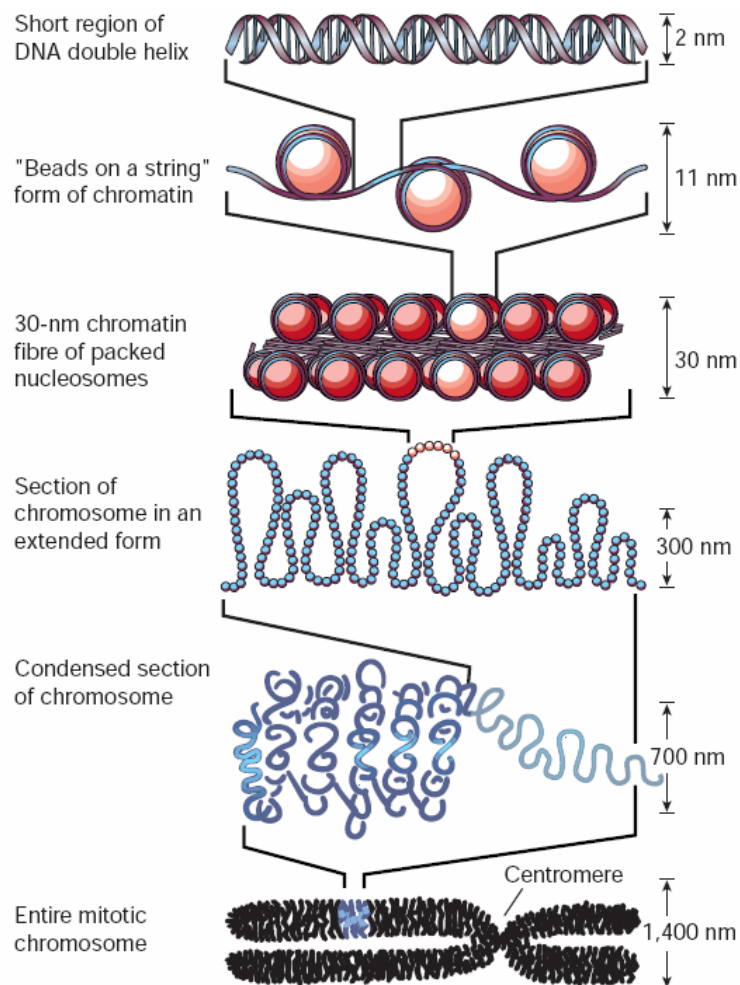


Figure 1 Packaging DNA

Eukaryotic DNA is highly compacted into chromosomes. DNA wrapped around nucleosomes forms an 11 nm, "beads on a string", conformation. This structure allows short-range internucleosomal interactions, resulting in a 30 nm fibre. These fibres are further folded into higher-order structures, the details of which remain to be determined. This produces a highly organised and condensed DNA fibre as depicted in the mitotic chromosome.

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genetic sequence. This is important for regulating the expression of particular genes in different cell types, during the cell cycle and development.

Certain chromatin regions termed heterochromatin are particularly condensed and transcriptionally inactive. These regions are often tri-methylated at Histone H3K9, H3K27 and H4K20 (Pokholok *et al.*, 2005; Barski *et al.*, 2007; Schotta *et al.*, 2004, Karachentsev *et al.*, 2005). In contrast, regions termed euchromatin are less condensed and more accessible to the cellular machinery involved with gene activation/transcription, replication and DNA repair. The regions involved with gene activation/transcription are often tri-methylated at Histone H3K4, H3K36 and H3K79, while residues H3K9, H3K14 and Histone H4 are often acetylated (Pokholok *et al.*, 2005; Koch *et al.*, 2007; Roh *et al.*, 2005).

Acetylation is mediated by the histone acetyl transferase domain (HAT) present in multiprotein complexes such as PCAF/GCN5 which catalyse the transfer of an acetyl group from acetyl-coenzyme-A cofactor (acetyl Co-A) to a lysine residue. This transfer neutralises the basic charge of the lysine residue, destabilising and altering the histone-histone and histone-DNA interactions, thus causing both local and global changes in the chromatin fibre (Shogren-Knaak *et al.*, 2006). Recognition of acetylated residues is mediated by bromodomain modules present in many chromatin-associated proteins and HATs. Such modules are present in TAF_{II}250, a large subunit of the TFIID complex important for transcription initiation. In addition, the plant homeodomain of TAF3, a subunit of TFIID, is highly selective for and binds directly to H3K4me3 (Vermeulen *et al.*, 2007). Therefore, although the nucleosome appears to package eukaryotic DNA into the confinements of the cell nucleus, it acts as a key regulator in the dynamics of DNA.

These histone modifications are reversible. Histone deacetylases (HDACs) such as Rpd3p (reduced potassium dependency-3 protein) catalyse the removal of acetyl groups from lysine residues, which generally correlates with gene repression, while methylated lysine residues can be demethylated by lysine demethylases. Although the above modifications are often associated with a particular chromatin state (gene activation or repression), they have been associated with the other state (Berger, 2007). It is therefore important to consider histone modifications in concert with each another as well as their genomic context to understand the biological activity.

In addition to the covalent modification of histones, non-histone proteins and multiprotein complexes mediate the remodelling and repositioning of nucleosomes for cellular processes requiring DNA access. Current studies suggest nucleosome occupancy at promoters become depleted upon gene induction and replenished upon gene repression (Lee *et al.*, 2004, Yuan *et al.*, 2005). Nucleosome remodelling complexes such as SWI/SNF and RSC use ATP to alter histone-DNA interactions and may account for the observed nucleosome movement (Lusser and Kadonaga, 2003). Also, other proteins such as Heterochromatin Protein 1 (HP1) which functions mainly in transcriptional repression (de Wit *et al.*, 2007), and topoisomerases which function in the prevention, as well as the release of DNA superhelical torsion, all play a part in ensuring the tightly packed eukaryotic genome is readily accessible to support the cells needs.

This project investigated the role of nucleosome binding protein HMGN3a in regulating gene expression. HMGN proteins belong to a family of non-histone proteins known as high mobility group proteins (HMG) and participate in the dynamics of chromatin. Based on a characteristic functional sequence, the HMG proteins are categorised into three subfamilies, these being HMGA, HMGB and HMGN (reviewed by Bianchi and Agresti, 2005). HMGA proteins bind AT rich sequences in the minor groove of DNA and regulate transcription, while HMGB proteins contain HMG-box domains that bind the minor groove of DNA and bend the DNA, promoting binding of other proteins like TATA binding protein (TBP). HMGN proteins bind to the nucleosome and affect the compactness of chromatin (Ding *et al.*, 1997). Binding of HMGN is associated with open, transcriptionally active chromatin and is reviewed by Bustin (Birger *et al.*, 2003, Bustin, 2001).

Much of the information regarding the HMGN proteins has been gathered from studies of HMGN1 and HMGN2. This investigation aimed to investigate the role of HMGN3 in regulating gene expression by studying the chemokine gene CCL1 in mouse embryonic fibroblast cells (MEFs).

Previously, RNase protection assays showed CCL1, along with other chemokines, were expressed in MEFs (West, unpublished data). To investigate whether the chromatin remodelling protein HMGN3a altered CCL1 expression, immortalised HMGN1^{-/-} MEFs were engineered to express HMGN3a under the Tet-On system.

HMGN3a is expressed on addition of doxycycline. Stimulating these MEF cells with cytokines such as TNF α induced expression of the chemokines identified by RNase protection, with the most reproducible results obtained for the expression of CCL1 following TNF α stimulation (West, unpublished data). QRT-PCR analysis showed HMGN3a reduced the level of TNF α -induced CCL1 expression (West, unpublished data). However, the mechanism by which HMGN3a mediates CCL1 expression is unknown.

To investigate the role of HMGN3 in regulating CCL1 gene expression, QRT-PCR was used to quantify CCL1 expression in the presence and absence of HMGN3, and chromatin immunoprecipitation (ChIP) to identify any histone modifications associated with HMGN3 across the CCL1 gene. As HMGN proteins bind to nucleosomes and affect cellular processes such as repair, transcription and during development, altered expression may have profound consequences and lead to abnormalities and disease (Hock *et al.*, 2007). Further characterisation of the HMGN proteins will improve the understanding for the mechanisms involved in controlling DNA-dependent activities in the cell and the role non-histone proteins play in regulating chromatin structure.

1.1 The HMGN family and functional domains

The HMGN proteins are approximately 10 kDa in size and found only in vertebrates. The two founding members of the HMGN family, HMGN1 and HMGN2, were isolated 26 years ago (Johns, 1982). More recently, HMGN3 and HMGN4 were discovered (West *et al.*, 2001; Birger *et al.*, 2001). The HMGN proteins share highly conserved domains with one another and are highly conserved across species including mouse, frog, cow and human. Each protein has the characteristic nucleosome-binding domain (NBD), a bipartite nuclear localisation signal (NLS), and a chromatin-unfolding domain (CHUD) (Figure 2, adapted from West *et al.*, 2001) which is the least conserved among the domains. The majority of information on these proteins has come from studies involving the founding members, but much of what has been learned is likely to be applicable to the other proteins owing to the high degree of homology, especially within the known functional domains. For example, it has been shown homodimeric complexes of both HMGN1 or HMGN2 bind tightly to the nucleosome core particle (Sandeem *et al.*, 1980), both proteins enhance

acetylation of Histone 3 Lysine 14 (H3K14) (Ueda *et al.*, 2006), and both reduce the compactness of the chromatin structure (Trieschmann *et al.*, 1995).

	NLS1	NBD	
N3a	<u>PKRK</u> SPENTEGKDGSKVT <u>KQEP</u> TRRSARLSAKP <u>PAP</u> PKPEPK <u>PRK</u> TSAKKEPGAKISRGA		
N3b	<u>PKRK</u> SPENTEGKDGSKVT <u>KQEP</u> TRRSARLSAKP <u>PAP</u> PKPEPK <u>PRK</u> TSAKKEPGAKISRGA		
N1	<u>PKRK</u> V-SSAEG-----AAKEEPKRRSARLSAKP-PAKVEAKPKKAAAK-DKSSDKKVQT		
N2	<u>PKR</u> KAEGDAKG-DKAKV-KDEPQRRSARLSAKP <u>PAP</u> PKPEPKPKKAPAK-----KGEKVP		
N4	<u>PKR</u> KAKGDAKG-DKAKV-KDEPQRRSARLSAKP <u>PAP</u> PKPEPRPKKASAK-----KGEKLP		
	NLS2	CHUD	
N3a	<u>KGK</u> KEEKQEAGKEGTA-----PSENGETKAEFAQKTESVDNEGE		98
N3b	<u>KGK</u> KEEKQEAGKEGTEN		76
N1	<u>KGK</u> RGAKGK-QAEVANQETKEDLPAENGETKTEESPASDEAGEKEAKSD		99
N2	<u>KGK</u> KG-KADAGKEGNN-----PAENGDAKTDQAQKAEGAGDAK		89
N4	<u>KGK</u> RG-KADAGKDGNN-----PAKNRDASTLQSQAEGTGDAK		89

Figure 2 Amino acid sequence alignment of human HMGN proteins

Conserved residues between HMGN members are in bold. Residues conserved between three of the full-length transcripts are underlined. The two nuclear localisation signals (NLS1 and NLS2), the nucleosome-binding domain (NBD) and chromatin-unfolding domain (CHUD) are highlighted in grey and indicated above the alignments. The total number of amino acids for each peptide is shown at the end of each sequence. (Adapted from West *et al.*, 2001)

1.2 HMGN genes

HMGN1, 2, 3 and 4 are present at positions 21q22.3, 1p36.1, 6q27, and 6p21.3 in humans, with the genes spanning 6.8, 3.5, 32 and 2kb respectively (Landsman *et al.*, 1989; Popescu *et al.*, 1990; West *et al.*, 2001; Birger *et al.*, 2001). The location of HMGN1, 2, and 3 in mouse lie at positions 16 69.8 cM, 4 62.2 cM and 9 E3.1, while further analysis is required to locate the HMGN4 gene. The genes for HMGN1, 2 and 3 have six exons, while HMGN4 is intronless. HMGN3 is much larger than the other HMGN genes and is largely due to intron I of HMGN3 spanning 18kb (West *et al.*, 2001).

1.3 HMGN3 transcription

The HMGN3 transcript is unique amongst the HMGN family in that it undergoes alternative splicing. This arises due to a 41-nucleotide deletion after the codon for glycine 72, causing a frameshift. As a result, the following codons encode Thr-Glu-Asn-STOP. HMGN3a denotes the full-length protein while HMGN3b the truncated protein. Although HMGN3a and HMGN3b are identical in sequence (to glycine 72), HMGN3b lacks the majority of the CHUD present in HMGN3a (Figure 2). This may have important implications in protein function, for both isoforms retain the capacity to associate with nucleosomes through the NBD, but only HMGN3a retains the functional CHUD. As such, HMGN3b may function as a competitive inhibitor to HMGN3a (or other family members) through nucleosome occupancy, or indirectly through dimer formation (HMGN3a-HMGN3b) as dimers exist between two HMGN1 or two HMGN2 proteins (Sandeem *et al.*, 1980).

1.4 Nucleosome binding domain

The highly conserved NBD is 30 amino acids in length and positively charged. The domain is lysine and proline rich (Figure 2). The NBD is the major determinant for HMGN protein interaction with the nucleosome, although FRAP analysis shows the C-terminal region contributes too (Ueda *et al.*, 2008). Embedded within the NBD is the conserved sequence RRSARLSA, residues 22-29 in HMGN2 (Figure 2). Mutational analysis of this sequence indicates four amino acids to be essential to permit nucleosomal binding, these being R₂₂ S₂₄ R₂₆ L₂₇ in HMGN2 (Ueda *et al.*, 2008). S24 of HMGN2 specifically binds to Histone H3,

indicating the NBD lies very close to Histone H3. The authors also report wild type HMGN2 has a similar affinity for purified DNA (deproteinised DNA) as a HMGN2-S24, S28 mutant. However, the mutant has a significantly decreased affinity for nucleosome core particles than wild type, suggesting HMGN proteins may be able to interact with chromatin without occupying nucleosomes (see Section 1.9).

1.5 Chromatin unfolding domain

Binding of the NBD to the nucleosome positions the C-terminal domain within the vicinity of the N-terminal tail of Histone H3 (Trieschmann *et al.*, 1998). Crosslinking of HMGN1 to nucleosome-DNA complexes showed the CHUD targets the N-terminal tail of Histone H3, thus, being ideally placed to interact with the tail domain of Histone H3. Such interaction could induce structural changes in the chromatin fibre directly by disrupting nucleosome-DNA or nucleosome-nucleosome interactions, and/or alternatively, through altering the ability of histone enzymes to modify the histone tails. Therefore, HMGN binding to nucleosomes may affect chromatin structure both at the individual nucleosome level and, in doing so, at the chromatin fibre level too.

With the exception of HMGN3b, all HMGN CHUDs are negatively charged. HMGN3a has the greatest negative charge of negative eight. The CHUD is responsible for transcriptional enhancement by HMGN proteins (Trieschmann *et al.*, 1995) and therefore HMGN3b may have a reduced impact on transcription (see Section 1.10).

1.6 Regulation and expression of HMGN proteins

Studies on the regulation and expression of HMGN proteins to date have focused on HMGN1, 2 and 3. These genes appear like most other housekeeping genes in that they have CpG islands that span the promoter region. However, there is no significant common regulatory element amongst the HMGN genes. A CCAAT sequence that binds the NF-Y transcription factor is present on HMGN1, 2 and 3, although this is found in many promoters (West *et al.*, 2001). A TATA box is present for HMGN2 and 4, but not HMGN1 or 3, while SP1 sites are present for HMGN1, 2 and 4 but absent from HMGN3. The absence of conserved

transcription factor binding sites amongst the HMGN genes suggests gene expression is regulated differently. This may account for the tissue specific expression profiles.

HMGN3 expression levels vary between tissues and between the same tissue types from different species (West *et al.*, 2001). HMGN mRNA levels within any particular tissue generally correlates with the level of protein. Human HMGN3 mRNA levels are highest in the pancreas, pituitary and heart while mice have highest levels in eye, brain and prostate. In contrast, human brain and prostate have low levels of HMGN3 and mouse has low levels in pancreas. Large differences in the level of HMGN3 between human and mouse brain may be due to the section of brain tissue analysed, as different regions of the brain had different levels of HMGN3 protein (Ito and Bustin, 2002). Nevertheless, between species many tissues have similar levels of mRNA expression, including lung, thyroid and spleen.

HMGN3b (Trip7) has a developmental role in *Xenopus laevis* (Amano *et al.*, 2002). In tissues undergoing metamorphosis, HMGN3b is expressed and up-regulated by Thyroid hormone, with the exception of tissues that undergo complete resorption. HMGN3b interacts with the thyroid hormone receptor and in the presence of thyroid hormone promotes transcription from chromatin templates. Pre- and post-metamorphosis, HMGN3 expression is low, but at the climax of metamorphosis, HMGN3 levels are high.

In a similar theme, during embryogenesis, expression of HMGN1 is progressively down-regulated (Furusawa *et al.*, 2006). Higher levels of HMGN1 expression occur in 7-day-old mouse embryos than in 11-, 15-, and 18-day-old embryos. HMGN1 expression is significantly decreased in differentiated tissues, although cells poised for renewal such as the basal layer of the stomach lining remain high. Similarly, HMGN2 is down-regulated in differentiated cells, but abundant in those undergoing active differentiation (Lehtonen and Lehtonen, 2001). This provides strong support that HMGN proteins play a significant role in differentiation and development. As has been suggested, this is likely to involve chromatin interaction, as re-expression of HMGN1 in HMGN1 knock out mice restores the rate of differentiation (of mesenchymal cells), whereas a HMGN1 mutant unable to bind chromatin, does not (Furusawa *et al.*, 2006).

1.7 Targeting of HMGN proteins to nucleosomes

Although it is known HMGN proteins bind to nucleosomes, it is unclear as to how targeting is accomplished as studies with HMGN1, 2 and 3 show the proteins have no preferential binding sequence (Shirakawa *et al.*, 2000; West *et al.*, 2001). HMGN1 and 2 have a slight preference for nucleosomes in DNA regions depleted of nucleotides CG and enriched in AT and TA, while methylation of CG sequences has no effect on HMGN binding (Shirakawa *et al.*, 2000). However, based on the affinity constants of HMGN proteins, this on its own is deemed insufficient to constitute a major factor in HMGN targeting.

HMGN proteins compete with linker Histone H1 for nucleosome binding sites (Catez *et al.*, 2002). Histone H1 proteins help stabilise chromatin at the individual nucleosome level and at the 30 nm fibre (Figure 1). Like HMGN proteins, these are dynamic and reside longer on than off the chromatin fibre. (Phair *et al.*, 2004). HMGN proteins compete for binding sites but do not displace bound Histone H1 from chromatin (Ueda *et al.*, 2008).

1.8 Gene regulation by HMGN proteins

The HMGN proteins bind transiently to chromatin. Moving rapidly throughout the nucleus (Catez *et al.*, 2003), the proteins “hop” on and off the chromatin fibre, spending longer on than off (Phair *et al.*, 2004). In some cases, HMGN proteins may reach their targets in complex with other proteins. Lim *et al.*, (2002) identified five HMGN1 and three HMGN2 containing complexes. More recently, HMGN2 was identified in complex with the paired-like homeodomain transcription factor 2 (PITX2) (Amen *et al.*, 2008). This prevents PITX2 from binding DNA. However, in the presence of Wnt signalling, B-catenin translocates into the nucleus and forms a complex with HMGN2-PITX2. This enables DNA binding of PITX2, activating transcription. In the absence of Wnt signalling, B-catenin is degraded in the cytoplasm, preventing the HMGN2-PITX2 complex binding its DNA target. This mechanism provides one possibility of how different HMGN family members are targeted to specific chromatin sites to regulate individual genes. Further, HMGN proteins are known to increase the efficiency of transcription initiation and in promoting transcriptional elongation by reducing the compaction of the chromatin fibre (Paranjape *et al.*, 1995; Ding *et*

et al., 1994). Therefore, HMGN proteins may fine-tune the targeting of transcription factors.

1.9 Cell cycle dependent localisation of HMGN proteins

The intranuclear distribution of HMGN proteins is cell cycle dependent. Several residues of the HMGN proteins undergo covalent modification including acetylation (Bergel *et al.*, 2000; Herrera *et al.*, 1999) and phosphorylation (Louie *et al.*, 2000; Lim *et al.*, 2004). During interphase, HMGN proteins are associated with chromatin as well as scattered throughout the nucleus. However, during the mitotic phase, the NBD undergoes phosphorylation, preventing nucleosome binding (Hock *et al.*, 1998; Prymakowska-Bosak *et al.*, 2001) and HMGN proteins are found scattered throughout the intracellular space as separate entities from chromatin. A decrease in the level of phosphorylated HMGN in the cytoplasm occurs with the progression of telophase, with a concomitant increase in non-phosphorylated HMGN bound to DNA in the nucleus (Hock *et al.*, 1998; Prymakowska-Bosak *et al.*, 2002).

However, more recently fluorescently labelled HMGN proteins were found associated with mitotic chromosomes (Pallier *et al.*, 2003). This contradiction has been investigated (Cherukuri *et al.*, 2008). HMGN1 residues S20 and S24 are critical for the specific nucleosome binding activity during interphase (Prymakowska-Bosak *et al.*, 2001); however, mutation to glutamic acid abolishes this. Pallier and co-workers show the mutant protein is in fact able to bind DNA during the mitotic phase, but this interaction is weaker and the protein unable to form specific complexes with nucleosomes, in contrast to interphase. Thus, this study established the NBD is not required for DNA binding during the mitotic phase (Cherukuri *et al.*, 2008).

The different cellular distributions of phosphorylated and unphosphorylated HMGN proteins during the interphase/mitotic transition may be mediated by 14.3.3 proteins, a class of protein involved in cellular targeting (Prymakowska-Bosak *et al.*, 2002). The phosphorylated HMGN NBD promotes binding to 14.3.3 proteins, impairing nuclear import of the protein. The proteins reside in the cytoplasm until the nuclear membrane reforms, thus reducing the level of chromatin bound HMGN during mitosis. Conceivably this is important to prevent

unwanted and unnecessary DNA transcription, as well as to promote a more condensed chromatin structure. This may enhance the ease at which the newly synthesised chromosomes are separated by the microtubules and may reduce the chance of erroneous events occurring such as chromosome breakages and translocations.

Once the nuclear membrane reforms the HMGN proteins re-associate with chromatin, mediated by active transport (Hock *et al.*, 1998). In late telophase, the HMGN proteins in a complex containing importin- α transport the proteins through the nuclear pores into the nucleus (Hock *et al.*, 1998). The proteins are then ideally placed to re-associate with the chromosomes.

1.10 Gene regulation by HMGN3

HMGN3 gene regulation studies show both HMGN3a and HMGN3b participate in the up and down regulation of gene expression in various cell types (West *et al.*, 2004a). Of the genes studied, none are up-regulated by both isoforms, although some are down-regulated by either. HMGN3a increases expression of the glycine transporter 1 gene (*Glyt1*), a gene expressed in the brain, by as much as 19-fold, while HMGN3b has a smaller effect. This is in agreement with studies in which mutant HMGN proteins lacking portions of their C-terminal domains conferred reduced transcription rates over full-length HMGN proteins. As the HMGN3b sequence is identical to the corresponding HMGN3a amino acid sequence (Figure 2), nucleosome binding via the NBD may induce a structural change in the chromatin fibre favouring an increase in transcription. However, HMGN3a may enhance the level of transcription further than HMGN3b by inducing histone modifications that favour a more stable chromatin conformation than that alone of HMGN3b, possibly through the CHUD interacting with the Histone H3 N-terminal tail, similar to that of HMGN1 (Section 1.5, Trieschmann *et al.*, 1998).

HMGN3 proteins are enriched around a 3.5 kb region of the *Glyt1* transcription start site, suggesting HMGN3 may play a role in enhancing transcription. HMGN3 proteins may bind some of the nucleosomes via their NBD in this 3.5 kb region and make available previously inaccessible nucleosomes. This may allow access of other histone modifying enzymes that may even be recruited and travel along

with polymerase II (Barski *et al.*, 2007), thus enhancing the efficiency of elongation by RNA polymerase.

As mentioned earlier, although HMGN3b enhances transcription to a lesser extent than HMGN3a, it can interact with the thyroid hormone receptor to promote thyroid-hormone-dependent transcription (Amano *et al.*, 2002).

Therefore, although similarity exists between HMGN proteins, each may interact differently with the same transcription factors (or different factors), conferring different properties on the DNA sequence they regulate. HMGN3a may act cooperatively with a transcription factor at the *Glyt1* gene, while HMGN3b acts less so.

1.11 Covalent post-translational modifications

Nucleosomal bound HMGN1 impedes MSK1 (mitogen-and stress-activated protein kinase) and RSK2 (ribosomal S6 kinase) mediated phosphorylation of Histone H3 serine 10 (Lim *et al.*, 2004). H3S10ph is correlated with transcriptionally inactive, condensed mitotic chromosomes but also with actively transcribed genes during interphase and during the transient expression of immediate early (IE) genes (Johansen and Johansen, 2006). IE genes are rapidly expressed following stimulation with growth factors, cytokines or pharmacological compounds such as anisomycin (reviewed by Thomson *et al.*, 1999a). HMGN1 S6 and H3S10 undergo rapid and transient phosphorylation in cells induced with anisomycin, an effect termed the nucleosomal response (Thomson *et al.*, 1999b). This is due to the highly efficient phosphorylation by MSK1 and to a lesser extent RSK2 (Thomson *et al.*, 1999b; Lim *et al.*, 2004). MSK1 also targets HMGN1 NBD at S20 and S24 (Lim *et al.*, 2004). When these residues are phosphorylated, HMGN is unable to bind the nucleosome (Figure 3A) (Prymakowski-Bosak *et al.*, 2001). During the nucleosomal response, phosphorylation of HMGN1 S20 and S24 precedes that of S6. This reduces nucleosome binding of HMGN1, allowing access of MSK1 to phosphorylate Histone H3S10 and S28 (Lim *et al.*, 2004).

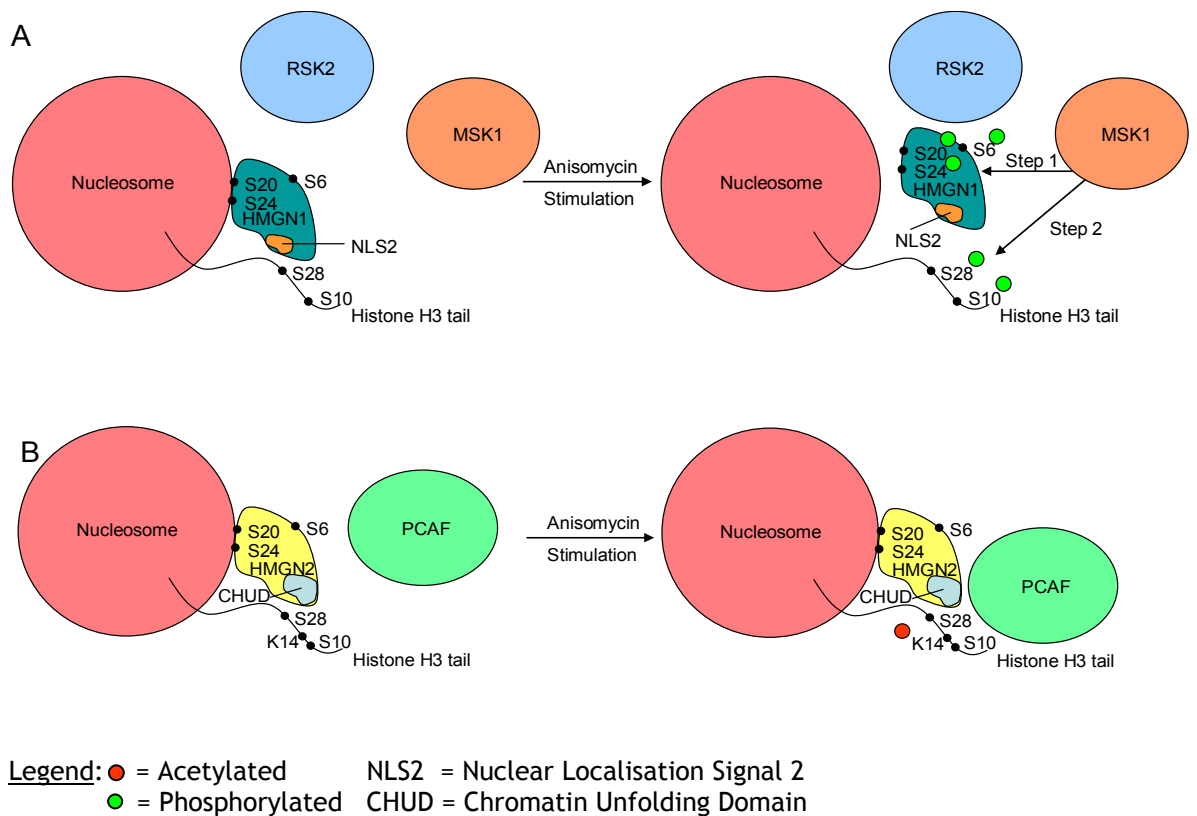


Figure 3 HMGN1 and HMGN2 modulate post-translational modifications of the nucleosome.

(A) HMGN1, but not HMGN2, inhibits MSK1 and RSK2 phosphorylation of Histone H3S10 and H3S28. Upon anisomycin stimulation, MSK1 phosphorylates HMGN1 serine residues S6, S20 and S24, step 1. HMGN1 loses interaction with the nucleosome, allowing MSK1 to phosphorylate Histone H3S10 and H3S28. Inhibition of MSK1 by HMGN1 is conferred by nuclear localisation signal 2 (NLS2). (B) HMGN2 enhances PCAF mediated acetylation of Histone H3K14. Enhancement is associated with the chromatin-unfolding domain (CHUD).

Unlike HMGN1, HMGN2 does not inhibit phosphorylation of H3S10 and S28 by MSK1 despite having a similar affinity constant for nucleosomes, indicating that binding alone is insufficient (Ueda *et al.*, 2006; Shirakawa *et al.*, 2000). Inhibition localises to residues 42-73 of HMGN1 (Ueda *et al.*, 2006).

HMGN2 does, however, enhance PCAF-mediated acetylation of H3K14 3.5-fold, compared with a significantly less 2-fold increase for HMGN1 (Ueda *et al.*, 2006, Lim *et al.*, 2005). Enhancement localises to residues 68-89 of HMGN2 which lie within the CHUD (Figure 2).

The CHUD motifs of HMGN1 and 2 have net negative charges of two and seven. The seven amino acid sequence conserved between HMGN2, 3 and 4 is less conserved for HMGN1 (Figure 2, HMGN1 residues 55-64) and may account for the different acetylation enhancements. As the HMGN3 isoforms have different overall C-termini charges they are likely to have different functions in chromatin modification.

1.12 Further HMGN functions

Incorporation of HMGN2 into chromatin during chromatin assembly stimulates replication efficiency of DNA (Vestner *et al.*, 1998). HMGN proteins are also involved in DNA repair. HMGN1 null mice appear normal but are hypersensitive to UV-B irradiation and have higher incidence of sporadic tumours (Birger *et al.*, 2003, West *et al.*, 2004b). It is likely HMGN1 opens up the chromatin structure, allowing the nucleotide excision repair machinery access to the site of DNA damage. The possibility HMGN1 affects remodelling of chromatin by the SWI/SNF complex proved negative, with HMGN1 having no apparent effect on the rate of remodelling (Hill *et al.*, 2005).

1.13 Alternative and diverse roles of HMGN

HMGN proteins are typically located within the nucleus, however, recently HMGN2 was found in the cytoplasm of IL-2 stimulated mononuclear leukocytes (Feng *et al.*, 2005). The NBD of HMGN2 was found to have strong antimicrobial properties to *E. coli* ML-35p. Thus, HMGN proteins may have roles in addition to

regulating chromatin structure; they may participate in immune defence, possibly through combat with invading bacteria.

The NBD has also been found to preferentially target tumour cells (Porkka *et al.*, 2002). The NBD of HMGN2 binds to and accumulates in the nuclei of mice bearing either HL-60 (human myeloid leukaemia cell line) or MDA-MB-435 (human breast carcinoma cell line) tumours, and in tumour endothelial cells. Nucleolin is responsible for shuttling the NBD from the cell surface into the nucleus (Christian *et al.*, 2003). Nucleolin is expressed mainly on the surface of cells undergoing proliferation and on angiogenic endothelium.

In one other, more recent study, a recombinant chimeric toxin using the NBD of HMGN2 and pseudomonas exotoxin domain III (PE) was generated (Xiong *et al.*, 2008). PE is an exotoxin derived from *Pseudomonas aeruginosa* that kills animal cells by inhibition of protein synthesis. *In vitro*, the cytotoxicity of the chimeric protein has a greater effect on HeLa cells than on normal human cervical epithelial cells, producing an increased mortality rate. Higher levels of the chimeric protein labelled with fluorescein isothiocyanate (FITC) were found in HeLa cells (31% of cells fluorescent) compared with almost no fluorescence in human cervical epithelial cells. *In vivo* experiments on mice bearing HeLa tumours treated with the chimeric protein show inhibition of tumour growth, large areas of necrosis and some apoptosis, with no obvious abnormalities in the other tissues or organs.

The studies by Porkka and Xiong *et al.*, provide evidence that HMGN proteins, or at least the NBD sequence that is characteristic of the HMGN proteins, have alternative roles from nucleosome binding. Further characterisation of HMGN proteins will enhance our understanding of gene regulation and chromatin dynamics. It may improve our ability to combat disease such as cancer by the direct delivery of anticancer compounds to cancerous cells using the NBD as a carrier. Alternatively, one family member of the HMGN proteins may be solely responsible for the up-regulation of an oncogene or down-regulation of tumour suppressor genes. Thus, it may be possible to target uniquely this member by, for example RNAi (Hill and Imbalzano, 2006), to suppress the undesirable effects if the other HMGN proteins can compensate for the normal regulation within healthy cells (HMGN1^{-/-} mice display only a mild phenotype (Birger *et al.*, 2003)).

1.14 Summary of HMGN proteins

HMGN proteins are non-histone proteins found only in vertebrates. These proteins bind to nucleosomes and DNA, reduce the compactness of chromatin and enhance transcription and replication from chromatin templates. They affect histone modifications, and in vivo, are highly dynamic and compete with Histone H1 for nucleosome binding sites. Cellular localisation of HMGN proteins are cell cycle dependent and they form complexes with other proteins. The NBD has the potential for use as a carrier to deliver drugs to cancerous cells.

2 CCL1

2.1 Introduction to chemokines

The chemokines form a family of chemotactic cytokines, best known for their role in regulating the migration of different cells in the body. Around 50 chemokines have been identified in humans and most if not all are thought to have arisen from gene duplication and diversification. As a result, many are found clustered in defined chromosome regions (Zlotnik *et al.*, 2006 and Colobran *et al.*, 2007). The latest nomenclature revision of chemokines and chemokine receptors (Zlotnik and Yoshie, 2000) categorises chemokines into four subfamilies based on the spacing of the first two cysteine residues, and the receptors are named according to the subfamily of chemokine that binds. Thus, the cytokine subfamilies are CC CXC XC CX3C. Recently a fifth subfamily was discovered in zebrafish and termed CX (Nomiya *et al.*, 2008). mCCL1 belongs to the CC subfamily.

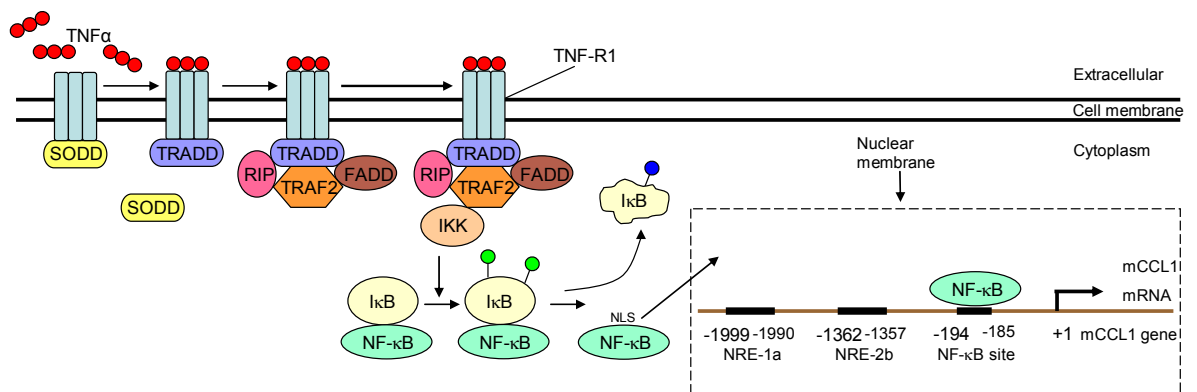
2.2 CCL1 gene structure

This project focuses on how HMGN3 participates in the regulation of T-Cell Activation gene 3 (TCA3). TCA3 (referred from here on in as mCCL1) is a murine chemokine originally isolated from a cDNA library derived from a T-cell clone (Burd *et al.*, 1987). The human homologue hCCL1 (also known as I-309) was identified soon after (Miller *et al.*, 1989). mCCL1 is located on mouse chromosome 11 47.32 cM and hCCL1 on human chromosome 17q11.2 (Burd *et al.*, 1987; Miller *et al.*, 1990). These genes are found clustered with the majority of other CC chemokines. A common theme amongst CC chemokines is that they have three exons with highly conserved intron/exon boundaries (Miller *et al.*, 1990, Zlotnik *et al.*, 2006). This suggests the genes evolved from a common ancestor. Human and mouse CCL1 share 55% nucleotide homology (Miller *et al.*, 1989) although greater homology exists 5' to the transcription start site (TSS). mCCL1 region (-320 to -76) and hCCL1 region (-314 to -91) share 69% homology (Miller *et al.*, 1990) and contain various conserved sequence motifs. These include a palindromic NF- κ B, E1A enhancer core and a PU.1 transcription factor binding site. In addition to these, hCCL1 contains an Ig H

chain enhancer octamer (-455 to -448) and a μ E5 element (-384 to -376). The NF- κ B element is important for regulating mCCL1 induction (Oh and Metcalfe, 1994) but further experiments are required to show whether any of the other elements participate in regulating gene expression. Initiation of mCCL1 transcription requires two intracellular signals for the efficient expression in T-cells (Wilson *et al.*, 1988). These signals are transduced through the T-cell receptor (TCR) complex, and like other lymphokine genes, this causes both an increase in intracellular calcium concentration and activation of protein kinase C (PKC).

2.2.1 NF- κ B element

The region extending 82 nucleotides upstream of the mCCL1 TSS induces reporter gene expression that is further enhanced by the region extending to -1342 (Oh *et al.*, 1997). An inducible NF- κ B element lies at position -194 to -185. Activation of the NF- κ B pathway by tumour necrosis factor alpha (TNF α) is well established and regulates the expression of other chemokines too. The TNF-induced activation of the NF- κ B signalling pathway involves TNF binding the transmembrane cell surface receptor TNF-R1, releasing the inhibitor protein SODD from TNF-R1's intracellular domain (Figure 4). This allows TRADD to associate with TNF-R1 and recruit RIP, TRAF2 and FADD. Other proteins are recruited here including IKK. IKK consists of two kinases, IKK α and IKK β , as well as a regulatory subunit IKK γ (reviewed by Ghosh and Hayden, 2008). Stimulation with TNF α activates IKK β phosphorylation of I κ B, leading to the degradation of I κ B and exposure of the NLS on NF- κ B. NF- κ B is free to translocate from the cytoplasm to the nucleus where it can bind NF- κ B elements and stimulate transcription. However, IKK α translocates to the nucleus and directly phosphorylates H3S10 and enhances H3K14ac (Anest *et al.*, 2003; Yamamoto *et al.*, 2003). CBP and IKK α are recruited to NF- κ B dependent promoters following TNF α stimulation and CBP acetylates H3K14.



Legend: TNF α , tumour necrosis factor α ; TNF-R1, TNF α -receptor 1; SODD = silencer of death domain; TRADD, TNF receptor-associated death domain; RIP, receptor-interacting protein; TRAF2, TNF-R-associated factor 2; FADD, Fas-associated death domain; I κ B, inhibitor of I κ B; IKK, I κ B kinase; NF- κ B, nuclear factor κ B; NLS, nuclear localisation signal; ●, phosphorylated; ●, ubiquitinated.

Figure 4 TNF α induction of mCCL1 expression through the NF- κ B pathway

The binding of TNF α to its transmembrane protein receptor induces the release of the inhibitor protein SODD from the intracellular surface of the transmembrane protein and the binding of TRADD. This leads to the recruitment of other intracellular proteins including IKK. In the inactive state, NF- κ B is sequestered in the cytoplasm by I κ B. I κ B bound to NF- κ B undergoes phosphorylation by IKK, leading to ubiquitination and degradation of I κ B, whilst exposing the NLS on NF- κ B. Translocation of NF- κ B to the nucleus and binding to the NF- κ B site induces transcription.

TNF α also stimulates the p38 pathway (Beyaert *et al.*, 1996). p38 induces H3S10 phosphorylation at a subset of NF- κ B dependent chemokine genes (Saccani *et al.*, 2002). Translocation of NF- κ B into the nucleus binds and activates genes that constitutively expose NF- κ B binding sites. p38 is thought to promote chromatin remodelling through phosphorylation of H3S10, thus, exposing less accessible NF- κ B binding sites. This way, depending on the magnitude of the stimulus (e.g. TNF α), the immune cell controls the level of response (e.g. recruitment of other immune cells - Section 2.3).

2.2.2 Negative regulatory elements (NRE)

Extending further from the TSS lie separate negative regulatory elements (NRE) (Oh *et al.*, 1997). These exist between -2057 and -1342 (Figure 4). This region can be subdivided into NRE-1 (-2057 to -1493) and NRE-2 (-1492 to -1342) based on a *Hind*III cleavage site. When placed in a reporter construct both elements inhibit CAT transcription in a distance (from TSS)-independent manner, but only NRE-1 is orientation-independent as NRE-2 only inhibited transcription when placed in the 5'-3' orientation. Either NRE inhibits transcription from CD20-promoter constructs, indicating a redundancy of NREs within the CCL1 promoter.

Within NRE-1 lie two sequences similar to the IL-4 silencer motif and are termed NRE-1a and NRE-1b. Only NRE-1a has been characterised and is essential for the regulatory activity of NRE1; however, it does not bind the protein that interacts with the IL-4 silencer sequence. Therefore, a novel protein may bind and confer the negative regulatory properties of NRE-1a.

Within NRE-2 lies a sequence similar to the lysozyme silencer motif, termed NRE-2a, and a sequence matching the silencer sequence of the α_{IIb} integrin gene, termed NRE-2b. NRE-2b is deemed necessary but not sufficient to confer the negative regulatory activity of NRE-2, and it may bind the same protein that interacts with the IL-4 silencer sequence though further purification of the identified protein complexes are required. NRE-2a does not bind to the same protein that interacts with the lysozyme silencer motif but may act synergistically with NRE-2b to confer negative regulatory activity.

To summarise the regulatory mCCL1 promoter sequences, the region extending 1342bp upstream from the TSS enhances transcription, while the region extending upstream from 1342 to 2057bp inhibits transcription (Figure 4).

2.3 CCL1 expression and secretion

mCCL1 is expressed in activated T helper 1 (T_H1), T helper 2 (T_H2), cytolytic T lymphocytes (CTL), natural killer T cells (NKT) and mast cells (Kennedy *et al.*, 2000; Wilson *et al.*, 1988). mCCL1 directly induces chemotaxis of monocytes/macrophages and neutrophils (Doyle and Murphy, 1999; Miller and Krangel, 1992; Luo *et al.*, 1994), while the human homologue hCCL1 is secreted by and is chemotactic for monocytes and T lymphocytes (Selvan *et al.*, 1997; Miller *et al.*, 1989; Miller and Krangle, 1992; D'Ambrosio *et al.*, 1998), but not neutrophils (Miller *et al.*, 1992). These differences/discrepancies may be due to different signalling mechanisms in mouse and human cells or due to experimental techniques. Initial studies showed mCCL1 was unable to induce chemotaxis of eosinophils (Doyle and Murphy, 1999). However, IL-5 overexpressing eosinophils elicited with TNF α (20 ng/ml) or high IL-4 concentration (20 ng/ml), induced migration and de novo CCR8 mRNA expression, the receptor for CCL1 (Oliveira *et al.*, 2002).

2.4 CCL1 binds CCR8

mCCL1 and hCCL1 share 42% amino acid homology (Miller *et al.*, 1989). The genes produce 92 and 96 amino acid proteins respectively, but the proteins contain a secretory signal sequence that is cleaved from the N terminus upon release (Burd *et al.*, 1987). The peptide undergoes asparagine-linked glycosylation that increases the molecular mass of the non-glycosylated peptide from 8 kDa to approximately 10 kDa (Luo *et al.*, 1994, Wilson *et al.*, 1990). Once secreted, the chemokine signals to cells expressing Chemokine Receptor 8 (CCR8), the unique receptor for CCL1 (Roos *et al.*, 1997; Tiffany *et al.*, 1997), although interaction with at least one additional receptor may occur (Goya *et al.*, 1998).

The murine and human CCR8 proteins share 71% homology (Goya *et al.*, 1998). CCR8 is a seven transmembrane domain G protein-coupled receptor and is highly

expressed in thymus, as well as spleen and colon (Zaballos *et al.*, 1996, Zingoni *et al.*, 1998; Goya *et al.*, 1998). mCCR8 is expressed on monocytes, CD4⁺ (helper) and CD8⁺ (cytotoxic) T lymphocytes (Tiffany *et al.*, 1997; Zaballos *et al.*, 1996; D'Ambrosio *et al.*, 1998). hCCR8 expression was shown to be specific to T_H2 lymphocytes and not T_H1 (Zingoni *et al.*, 1998). hCCR8 is also expressed on a subset of CD4⁺ memory T lymphocytes, enriched in T_H2 and T_{REG} cells, while only a small population of hCCR8⁺ T_H1 and NK cells exist (Soler *et al.*, 2006).

2.5 Associated disease

The chemokines are well known for their role in cell migration but they also play a role in disease. The dysregulation of chemokines is often associated with inflammation and tissue injury. mCCL1/hCCL1 has been implicated in asthma, atherosclerosis, diabetes and cancer and has both anti- and pro- apoptotic functions. Studying the murine homologue in vitro provided an opportunity to characterise how HMGN3 regulates gene expression and identify potential epigenetic marks associated with mCCL1 expression.

2.5.1 Asthma

Higher levels of hCCL1 are present in the bronchoalveolar fluid (BALF) of atopic asthmatic patients than in non-asthmatic people (Montes-Vizuet *et al.*, 2006). This correlates with an increase in lymphocytes and eosinophils (but not macrophages) and contributes to the inflammatory response. BALF, a substance coating the bronchial airway epithelial lining, contained a six-fold higher level of hCCL1 in asthmatics compared with normals. hCCL1 mRNA is constitutively expressed in bronchoalveolar cells in normal subjects but is much higher in asthmatics. This study found increased levels of hCCL1 in BALF correlates with an increased number of lymphocytes. In vitro assays show that bronchial epithelial cells release hCCL1 when stimulated by cytokines released by lymphocytes, such as IL-4 from T_H2 cells. The authors suggest hCCL1 released by the bronchoalveolar cells plays a role in lymphocyte recruitment in bronchial asthma.

A similar study in mice looked at the role of mCCL1 in the development of allergic airway disease (Bishop and Lloyd, 2003). After allergen challenge,

macrophages were identified as the primary source of mCCL1 in BALF and lung tissue. Analysis shows allergen exposure causes an increase in both mCCL1 RNA and protein expression. mCCL1 mediates the recruitment of eosinophils, but not T_H2 lymphocytes. The lack of an apparent T_H2 recruitment in this study compared to that above may be due to the already increased number of T_H2 cells in BALF of mice. Alternatively, the allergen presented to mice may activate alternative pathways from those in atopic asthmatics and thus, these pathways exert a different overall effect on the recruitment of T_H2.

2.5.2 Atherosclerosis

The condition of atherosclerosis involves plaque formation, during which circulating monocytes associate with the vessel wall (Ross 1999). An independent risk factor for atherosclerosis called Lipoprotein(a) [Lp(a)] (Seman *et al.*, 1999) accumulates in blood vessel walls. Lp(a) prevents the binding of plasminogen, the inactive precursor of plasmin that functions in clot lysing (Hajjar *et al.*, 1989), and hence promotes plaque formation. Lp(a) also promotes recruitment of monocytes to the vessel wall (Poon *et al.*, 1997) and stimulates endothelial cells to produce hCCL1 (Haque *et al.*, 2000), a potent monocyte chemoattractant. This required de novo synthesis of hCCL1 and binding to hCCR8. Immunohistochemical studies show hCCL1 present in human atherosclerotic plaques and extracellular matrix, which colocalised with Lp(a). This suggests Lp(a) may function to recruit monocytes and other hCCR8 expressing cells, such as macrophages or lymphocytes, into the forming atherosclerotic plaque.

A subsequent study found hCCL1 and Lp(a)-stimulated endothelial cell-conditioned medium induced endothelial chemotaxis (Haque *et al.*, 2001). CCR8 and hCCL1 are present on the luminal endothelium of the atherosclerotic plaque and in subendothelial tissue of the atherosclerotic plaque. Endothelial chemotaxis was mediated through CCR8. Based on previous results that suggested new vessel formation accompanied plaque growth (Moulton *et al.*, 1999), the authors suggest chemotaxis of endothelial cells by hCCL1, accompanied by other inflammatory cytokines, promotes new vessel growth.

2.5.3 Cancer

Mouse myeloma cell lines J558 and P3X transfected with mCCL1 show impaired growth in both normal and immunodeficient mice (Lanig *et al.*, 1994). After 21 days of being tumour free, mice rechallenged 2-10 weeks later with nontransfected mCCL1 myeloma cells of the same myeloma cell line are resistant. Mice transfected with the other cell line are not, indicating antigen specific tumour immunity.

Injecting a mixture of mCCL1 transfected/nontransfected cells into mice produces retarded tumour growth, but an eventual tumour mass develops. In contrast, injection of transfected or nontransfected cells into opposite flanks of the mouse causes growth inhibition only in mCCL1 transfected tumours.

Further experiments show the mCCL1 anti-tumour effect is restricted to the early phase of tumour implantation, before a solid tumour develops, as mCCL1 failed to impair growth at the site of an established tumour.

Biopsies taken from mice injected with mCCL1 transfected myeloma cells contain high numbers of neutrophils, some monocytes and occasionally mast cells, 24 and 48 hours after implantation. Tumour necrosis occurs, but not in control experiments, nor an influx of leukocytes. Therefore, mCCL1 mediates inhibition of *in vivo* tumour growth by inducing an influx of immune cells that is likely mediated through interaction with the CCR8 receptor displayed by neutrophils, monocytes and mast cells.

In contrast to the pro-apoptotic functions described for myeloma, mCCL1 has anti-apoptotic functions in thymomas and may mediate positive selection of thymocytes (Zingoni *et al.*, 1998; Van Snick *et al.*, 1996).

An anti-apoptotic function for hCCL1 has been suggested for the progression of Adult T-cell leukaemia (ATL). ATL-derived lymphocytes highly overexpress and secrete hCCL1 and express CCR8 (Ruckes *et al.*, 2001). Cell culture supernatant of ATL cells dramatically reduces the rate of dexamethasone-induced apoptosis, but this can be overcome with the addition of anti-hCCL1 antibodies.

Similarly, Fas induced apoptosis of ATL cells is enhanced when anti-hCCL1 antibodies are added to the cell medium, while pertussis toxin, an inhibitor to the hCCL1 signalling, significantly increases apoptosis of ATL derived cells. This suggests hCCL1 participates in an anti-apoptotic autocrine loop that may inhibit apoptosis in ATL cells and contribute to their growth.

2.5.4 CCL1 concentration mediates cell response

Aside from the role of mCCL1 in the recruitment of monocytes/macrophages and neutrophils, neutrophils and monocytes produce cell-damaging chemicals (Devi *et al.*, 1995) which are associated with antitumour and antimicrobial action (Ding *et al.*, 1988).

Clearly, a coordinated series of events must ensue to control migration and release of harmful chemicals and enzymes to the appropriate target cells. This seems to be controlled by the concentration of mCCL1 in the environment of the target cell. At low concentrations (0.1nM), cell adhesion is induced and maintained (Devi *et al.*, 1995). As the concentration approaches 1nM, migration of cells including macrophages and neutrophils takes place. As the concentration increases to 10nM and higher, reactive oxygen and nitrogen intermediates are released by macrophages and neutrophils. This would explain the observed influx of monocytes and neutrophils observed in the study by Laning (Section 2.5.3), and account for the observed necrosis. At the site of implanting mCCL1 expressing myeloma cells, the concentration of mCCL1 would be greatest and induce necrosis, while non-transfected mCCL1 cells implanted at a flanking site would not.

2.5.5 Diabetes and other CCL1 associated disease

mCCL1 also participates in the clearance of *C. neoformans* from the lungs and brains of mice, a fungal pathogen acquired through inhalation that can spread to the brain causing meningoencephalitis, which is fatal if untreated (Idnurm *et al.*, 2005). mCCL1 attracts neutrophils to the site of infection which stimulate the production of mCCL3. This in turn promotes recruitment of lymphocytes into the reaction site and to promote clearance. A similar process of recruitment occurs in humans with the condition vernal keratoconjunctivitis (VKC), a disease

of the inner lining of the eyelids. Monocytes and macrophages express hCCL1 and recruit T lymphocytes which may contribute to the severity of VKC (El-Asrar *et al.*, 2002).

hCCL1 has also recently been characterised with a role in type 1 diabetes, involving macrophage recruitment and activation (Joseph Cantor and Kathryn Haskins, 2007).

Thus, the chemokine hCCL1 may be an important therapeutic target for people suffering from various conditions. Mouse and human studies of CCL1 will enhance our understanding of chemokine signalling in health and disease, and identify potential therapeutic targets to combat inflammation.

3 Aims

Previously, HMGN3 was shown to reduce TNF α -induced mCCL1 gene expression, however, it was unknown how HMGN3 brought about this effect.

The first aim was to confirm the previous studies finding that HMGN3 reduces TNF α -induced mCCL1 gene expression. The second aim was to study the effect serum concentration had on the level of mCCL1 expression. The third aim was to establish the optimal incubation time to induce *mCCL1* mRNA expression following TNF α stimulation.

To understand how HMGN3 regulates gene expression, ChIP assays were performed. The level of HMGN3 bound at the mCCL1 gene in cells induced with, or without, TNF α was measured to establish whether HMGN3 has a direct role in regulating gene expression. The level of acetylation at H3K14 was also investigated, as this mark is associated with transcribed genes. ChIP assays were quantified using QRT-PCR analysis.

Finally, to establish whether HMGN3 has an indirect role in regulating gene expression, NF- κ B and p38 luciferase assays were performed.

4 Materials and Methods

4.1 Materials

Agarose Multi Purpose Roche, Lewes, East Sussex, UK

Axygen 0.2 and 0.5 ml thin wall clear flat cap PCR tubes Thistle Scientific, Glasgow, UK

Axygen 1.5 ml and 1.7 ml MAXYMUM RECOVERY clear microtubes Thistle Scientific, Glasgow, UK

Barrier Tips (10, 20, 200, 1000 μ l) (RNase-DNase free, sterile) Neptune, USA

Bromophenol Blue Fisher Scientific, Loughborough, UK

DEPC-treated Water Invitrogen, Paisley, UK

Doxycycline SIGMA, Poole, Dorset, UK

Dual-Luciferase Reporter Assay System Promega, Southampton, UK

EDTA (Ethylenediaminetetraacetic acid disodium salt dehydrate) SIGMA, Poole, Dorset, UK

Ethidium Bromide Amresco, Ohio, USA

Ethyl Alcohol 99.7% v/v min Hayman Limited, Witham, Essex, England

FastStart SYBR Green Master (Rox) Roche, Lewes, East Sussex, UK

Formaldehyde 37% Solution Amresco, Ohio, USA

Glycerol BDH Laboratory Supplies, Poole, England

Glycine Fisher Scientific, Loughborough, UK

Glycogen (for molecular biology) Roche, Lewes, East Sussex, UK

Igepal CA-630 **SIGMA, Poole, Dorset, UK**

IgG (1 mg/ml) from rabbit serum **SIGMA, Poole, Dorset, UK**

Kb DNA Ladder **Stratagene, Amsterdam, The Netherlands**

LightCycler 480 Multiwell Plate 96 **Roche, Lewes, East Sussex, UK**

LightCycler 480 Sealing Foil **Roche, Lewes, East Sussex, UK**

Lithium Chloride **SIGMA, Poole, Dorset, UK**

MycoAlert Mycoplasma Detection Kit **Cambrex/Lonza, Wokingham, Berkshire, UK**

Optically Clear Heat Sealing Film **Abgene, Epsom, Surrey, UK**

Phenol:Chloroform:Isoamyl Alcohol 25:24:1 (Saturated with 10 mM Tris, pH 8.0, 1 mM EDTA) **SIGMA, Poole, Dorset, UK**

Protease inhibitor cocktail tablets (Complete, EDTA-free) **Roche, Lewes, East Sussex, UK**

Proteinase K from *Tritirachium album* **SIGMA, Poole, Dorset, UK**

Protein G Agarose, Fast Flow (10 ml packed beads) 20 ml 50% slurry **Upstate (Millipore), Wolverton Mill South, Milton Keynes, UK**

Random Hexamers (50 ng/ μ l) **Invitrogen, Paisley, UK**

Recombinant Mouse TNF α (Catalogue Number 410-MT-010/CF) **RnD Systems, Abingdon, Oxfordshire, UK**

Ribonuclease A from bovine pancreas **SIGMA, Poole, Dorset, UK**

RNase-Free DNase Set **Qiagen, Crawley, West Sussex, UK**

RNase H **Invitrogen, Paisley, UK**

RNaseOUT Recombinant Ribonuclease Inhibitor (40 U/ μ l) Invitrogen, Paisley, UK

RNeasy Mini Kit Qiagen, Crawley, West Sussex, UK

SafeViewTM Nucleic Acid Stain NBS Biologicals, Huntingdon, Cambridgeshire, UK

Sodium Chloride BDH Laboratory Supplies, Poole, England

SuperScriptTM III First-Strand Synthesis System for RT-PCR Invitrogen, Paisley, UK

SuperScriptTM III Reverse Transcriptase Invitrogen, Paisley, UK

SYBR[®] Green PCR Master Mix Applied Biosystems, Warrington, UK

Tris Base Fisher Scientific, Loughborough, UK

Triton X-100 Pharmacia Biotech, Uppsala, Sweden

Water (DNase-RNase free, 0.1 μ m filtered) SIGMA, Poole, Dorset, UK

0.1 M DTT (Dithiothreitol) Invitrogen, Paisley, UK

0.2 ml semi-skirted 96-well PCR plate (colour natural) Abgene, Epsom, Surrey, UK

1 KB Plus DNA Ladder Invitrogen, Paisley, UK

10 mM dNTP Mix (PCR Grade) Invitrogen, Paisley, UK

10x First Strand Buffer Invitrogen, Paisley, UK

25 mM MgCl₂ Invitrogen, Paisley, UK

100 bp DNA Ladder Invitrogen, Paisley, UK

4.1.1 Tissue culture plasticware and reagents

Cell lifter Corning, NY, USA

Centrifuge Tube (15 ml and 50 ml) Corning, NY, USA

DMEM + GlutaMAX-1 (1X) 32430 Invitrogen, Paisley, UK

DPBS - Dulbecco's Phosphate Buffered Saline without Ca and Mg (10x)

Lonza/Cambrex, Wokingham, Berkshire, UK

Hygromycin B in PBS 50 mg/ml Invitrogen, Carlsbad, CA, USA

LipofectaminTM 2000 Transfection Reagent Invitrogen, Carlsbad, CA, USA

Multidish 6 wells Nunc, Roskilde, Denmark

Pipette (1 ml, 5 ml, 10 ml, 25 ml, 50 ml) Corning, NY, USA

Tet-free USDA System Approved Foetal Bovine Serum Clontech, Saint-Germain-en-Laye, France

TC dish 100x20 SI, TC Dish 140x20 Nunc, Roskilde, Denmark

Trypan Blue Stain 0.4%, Opti-MEM (1X) Invitrogen, Paisley, UK

Zeocin Invitrogen, Carlsbad, CA, USA

0.25% Trypsin-EDTA (1x) Invitrogen, Paisley, UK

5 ml Polystyrene Round-Bottomed Tube (FACS tubes) BD Biosciences, Erembodegem, Belgium

50 ml Reagent Reservoir Corning, NY, USA

96 Well Assay Plate, (White Plate, Clear Bottom with Lid, Tissue Culture Treated, Polystyrene, Sterile) Corning, NY, USA

4.1.2 Antibodies

Anti-acetyl-Histone H3 (Lys14) (Rabbit, Polyclonal) Catalogue Number: 07-353
Upstate, UK

Mouse HMGN3 Ab: mN3Ab - EPO 62146

4.1.3 Primers

CCL1 genomic (QRT-PCR) primers (Table 1) Eurogentec, Southampton,
Hampshire, UK

Globin and Glyt1 genomic primers (Table 1), cDNA primers (Table 2) MWG
(Eurofins), Worples Park, London

4.1.4 Lab equipment

BIOHIT e120 automatic pipette Alpha laboratories, Eastleigh, Hampshire, UK

Thermocycler T3000 Biometra, Germany, Thistle Scientific

Thermo-sealer ABGene (Advanced Biotechnologies Ltd) Surrey, UK

LightCycler480 Roche, Mannheim, Germany

Microscope Olympus CK2 Olympus, Watford, England

Luminoskan Ascent Thermo Fisher Scientific, Ulm, Germany

Nanodrop ND-1000 Spectrophotometer Thermo Fisher Scientific, Ulm, Germany

Phosphoimager FLA-5000 Fujifilm, CT, USA

Plate Reader Dynatech MR7000 (Mycoplasma assays) Dynatech Laboratories
Incorporation, Guernsey, UK

Sonicator 3000 Misonix Incorporated, NY, USA

4.2 Buffers

4.2.1 General buffers

Phosphate buffered saline (PBS) (10x)

Isotonic buffer for washing cells

4.2.2 ChIP buffers

5% Formaldehyde Solution

5% CH₂O, 50 mM Tris pH 8.0, 0.1 M NaCl, 1 mM EDTA

1M Glycine solution

1 M Glycine

Cell lysis buffer

10 mM Tris pH 8.0, 10 mM NaCl, 0.2% Igepal CA-630. 1 protease inhibitor cocktail added per 10 ml prior to use.

Nuclei lysis buffer

50 mM Tris pH 8.0, 10 mM EDTA, 0.5% SDS

IP dilution buffer

12.5 mM Tris pH 8.0, 0.19 M NaCl, 0.01% SDS, 1.2% Triton X-100

IP wash I

20 mM Tris pH 8.0, 0.15 M NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100

High salt buffer

20 mM Tris pH 8.0, 0.5 M NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100

IP wash II

10 mM Tris pH 8.0, 0.25 M LiCl, 1 mM EDTA, 1% Igepal CA-630, 0.5% Sodium Deoxycholate

Tris-EDTA (TE) buffer

10 mM Tris pH 8.0, 1 mM EDTA

Elution buffer

1% SDS, 0.1 M NaHCO₃

4.2.3 Molecular biology solutions**DNA/RNA loading buffer**

0.25% (w/v) bromophenol blue, 10% (v/v) 1x PBS, 50% (v/v) glycerol in DNase-
RNase-free H₂O

4.3 Cell culture and treatments**4.3.1 Cell preparation**

Cell culture was performed in a Class II cabinet using sterile equipment and reagents. Cells previously aliquoted into cryo-tubes and stored in liquid nitrogen were thawed rapidly by placing in a 37°C water bath. Cells were immediately added to 9 ml fresh media, centrifuged at 155 x g for 4 minutes and supernatant removed to remove dimethylsulphoxide (DMSO). Cells were then resuspended in fresh media.

4.3.2 Mouse embryonic fibroblast T23 cells

Previously, SV-40-immortalised *Hmgn1*^{-/-} mouse embryonic fibroblast (MEF) cells were stably transfected with pTet-On, pSV-Zeo, pTK-Hyg, and the HMGN3 gene bearing tetracycline-responsive promoters. pTet-On expresses a tetracycline-controlled transactivator consisting of a bacterial Tet Repressor fused to three repeats of a minimal VP16 transcription activation domain. In the presence of Doxycycline (Dox), the transactivator activates transcription from the Tet promoter inducing HMGN3 expression. These cells (T23) are adherent and grow in monolayer.

4.3.3 Cell incubation conditions

T23 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 250 µl Zeocin and 800 µl Hygromycin. Cells were maintained in a humidified incubator of 95% air, 5% CO₂ at 34°C. 2 µg/ml Dox was added to the appropriate cells to induce HMGN3. Experiments where TNFα (0.05 µg/µl) was added, cells were incubated as described, however the 24 hours preceding TNFα induction the cell media was replaced with DMEM supplemented with only 0.1% FBS, no antibiotics, only Dox to the appropriate cells. At the time of adding TNFα, the media was replaced with the DMEM containing 0.1% FBS*, Zeocin and Hygromycin.

*The effect of using 0.1% and 10% FBS was investigated and shown in Figure 5.

4.3.4 Subculturing of cells

Upon reaching 80-95% confluency, cells were sub-cultured. Media was aspirated and cells washed with 10-20 ml Phosphate Buffered Saline (1x) for 2-3 minutes. After removing PBS, 2-3.5 ml of 0.25% Trypsin-EDTA (1x) solution was added until cells detached from dish. Then, 4-7 ml of fresh medium was immediately added to neutralise the Trypsin before sub-culturing cells to a new dish containing fresh media. The MycoAlert Mycoplasma Detection Kit was occasionally used to test for contamination.

4.4 Reverse transcriptase polymerase chain reaction

4.4.1 RNA extraction

Total RNA was extracted from 5×10^5 cells growing in six well plates using an RNeasy Mini Kit (Qiagen) following the manufacturers animal cell protocol. Cells were washed with PBS before commencing the protocol. Lysate was flash frozen on dry ice before storing at -80°C. Thawing of lysate from -80°C ensured adequate disruption and homogenisation of samples to produce sufficient RNA yields. The optional DNase digestion step was performed as per manufacturer's protocol. Samples were stored at -20°C or used immediately to synthesise first strand cDNA.

4.4.2 RNA gel electrophoresis

Total RNA samples were resolved by agarose gel electrophoresis to determine RNA integrity. Agarose gels (1-1.5%) were made by boiling agarose powder in 50 ml 1x TBE buffer in a microwave oven. Then, 1 μ l of ethidium bromide or 0.3 μ l SafeViewTM nucleic acid stain was added prior to casting the gel. The concentration of each RNA sample was determined using Nanodrop ND-1000, before 300 ng of RNA mixed with loading buffer was electrophoresed at 90-100 V for about 1 hour. A 1KB Plus DNA Ladder was loaded to clarify the size of ribosomal RNA bands. Images were taken using a phosphoimager. Clear and distinct 28S and 18S ribosomal RNA bands were visible on the gel image, indicating intact RNA samples. RNA was then reverse transcribed to produce cDNA.

4.4.3 First strand cDNA synthesis

cDNA was synthesised from total RNA using the SuperScriptTM III First-Strand Synthesis System for RT-PCR (Invitrogen). 300 ng of total RNA was added to a 0.2 ml thin walled PCR tube, together with 1 μ l random hexamers (50 ng/ μ l), 10 mM dNTP mix and made up to 5 μ l with DEPC-treated water. Samples were incubated at 65°C for 5 minutes then placed on ice for at least 1 minute. cDNA Synthesis Mix was prepared using half manufacturers recommended volumes. 1 μ l 10x RT (Reverse Transcriptase) buffer, 2 μ l 25 mM MgCl₂, 1 μ l 0.1 M DTT (Dithiothreitol), 0.5 μ l RNaseOUT (40 U/ μ l) and 0.5 μ l SuperScriptTM III RT (200 U/ μ l) were combined and 5 μ l added to each RNA/primer mixture. All samples were mixed gently before incubating for 10 minutes at 25°C, followed by 50 minutes at 50°C, 5 minutes at 85°C then 15 minutes at 4°C. RNase H (0.5 μ l) was added to each sample and incubated for 20 minutes at 37°C. The resulting 10.5 μ l cDNA samples were stored at -20°C.

4.4.4 QRT-PCR of cDNA

Quantitative Real Time-PCR (QRT-PCR) is a suitable method for comparing the level of gene expression in induced cells to those uninduced (HNGN3 induced/uninduced, CCL1 induced/uninduced). QRT-PCR reactions were performed using 0.21 μ l of cDNA diluted in 4.79 μ l of DNase-RNase free water

per 25 μ l reaction. 12.5 μ l of SYBR® Green PCR Master Mix (Applied Biosystems) (containing dNTP's, buffer, polymerase, SYBR green, Rox dye) together with 50 or 900 nM forward and reverse primers were added, with the final volume made up to 25 μ l with DNase-RNase free water. The primers used for QRT-PCR are listed in Table 2. GAPDH served as an internal control to normalise any differences in total cDNA input between samples that may arise due to efficiency of RNA purification, reverse transcription or from pipetting error. Samples were loaded to a 0.2 ml semi-skirted 96-well PCR plate (colour natural) using an automatic dispensing pipette, sealed, then centrifuged at 125 x g for 1 minute to collect samples before being loaded into a Stratagene MX3000P PCR machine. QRT-PCR reactions were carried out using the following conditions: 10 minutes at 95°C, followed by 45 cycles consisting of 15 seconds at 95°C and 1 minute at 60°C. Data was analysed using the Stratagene MX3000P v3.20 software. The reference dye Rox was used to normalise the minor differences present between samples well, such as differences in the volume of QRT-PCR reaction or transparency of Sealing Film. The $\Delta\Delta C_t$ method of analysis was carried out in excel to analyse the results.

4.5 Chromatin immunoprecipitation techniques

4.5.1 Fixation conditions

The following procedure was used to carry out ChIP.

T23 MEF cells were incubated as described previously in Section 4.3.3. Cells were grown on five 140 mm dishes to 80-90% confluency, with an additional plate reserved to count cells using a haemocytometer and to extract total RNA in some cases. Typically, 1×10^7 cells per 140 mm dish were used for each ChIP condition. Cells were placed on a rocker and fixed with 5% formaldehyde solution (10% of media volume (v/v)), then left for 5 minutes before fixations were quenched by addition of 2.25 ml 1 M glycine. Media was aspirated and cells washed in 20 ml ice-cold 1x PBS for 2 minutes. Cells were harvested by adding 8 ml ice cold 1x PBS and collected by scraping, then transferred to a 50 ml falcon tube, centrifuged at 220 x g for 5 minutes and supernatant removed. Cells were swelled/lysed using 5 pellet volumes (3.3 ml) of ice cold Cell Lysis Buffer, left on ice 10 minutes, centrifuged 5 minutes at 220 x g to pellet nuclei

and supernatant removed. Pelleted nuclei were lysed with ice cold Nuclei Lysis Buffer (2 ml), with the nuclei pellet disrupted by pipette action. Samples were either left on ice 10 minutes or frozen and stored at -20°C .

4.5.2 Sonication conditions

Chromatin samples were transferred from a falcon tube to a 5 ml FACS tube for sonication by a Misonix Sonicator 3000. The microtip was placed approximately $\frac{1}{3}$ of the way into the sample. A beaker containing ice was placed around the tube. The following settings were applied for sonication: 2 minutes on time: 10 seconds on, 30 seconds off, 6 watts, output three; i.e. each sample to be sonicated takes 8 minutes.

4.5.3 Preparation of DNA fragments for electrophoresis

A 100 μl aliquot of chromatin was combined in a tube with 6 μl 5 M NaCl_2 , 1 μl 1 mg/ml RNase and incubated at 65°C for 4 hours. Then, 3 μl 24.1 mg/ml proteinase K was added and incubated overnight at 45°C . The following day 100 μl Phenol:Chloroform was added, mixed, centrifuged at $14\,500 \times g$ for 5 minutes then the top layer containing DNA transferred to a new tube. This step was repeated to reduce the protein content. Having transferred the DNA to a new tube, 2 volumes 100% ethanol and $\frac{1}{10}$ volume 3 M Sodium Acetate were added and the sample incubated for 15 minutes at -20°C , then centrifuged at $18\,000 \times g$ for 15 minutes at 4°C . Supernatant was removed, 1 ml 70% ethanol added, mixed and centrifuged for 10 minutes at $18\,000 \times g$. Supernatant was removed and sample left for about 15 minutes at room temperature (RT) to air dry. Sample was resuspended in 100 μl distilled water.

4.5.4 DNA gel electrophoresis

DNA fragments were resolved by agarose gel electrophoresis to determine the size range of chromatin fragments produced after sonication. A 1.5% agarose gel was made by boiling agarose powder in 50 ml 1x TBE buffer in a microwave oven. Then 1 μl of ethidium bromide or 0.3 μl SafeViewTM stain was added prior to casting the gel. A DNA volume of 10 μl , mixed with 1.5 μl loading buffer and 2.5 μl DNase-RNase-free water were subjected to electrophoresis at 90-100 V for

about 1 hour. A 100 bp (1.5 μ l) and 1kB (2.5 μ l) marker mixed with 1.5 μ l loading buffer and made up to 15 μ l with DNase-RNase-free water were also added. Images were taken using a phosphoimager. Fragments in the range of ~400-1200 base pairs of DNA were ideally sought after and were visible as a smear on the gel.

4.5.5 Pre-clearing chromatin

After confirmation of the range of sonicated chromatin fragments, sonicated samples were pre-cleared. The samples were diluted in a 15 ml falcon tube with IP Dilution Buffer to a final volume of 4.5 ml. To this, 50 μ l (1 mg/ml) IgG (from rabbit serum) were added to each sample and left for 1 hour at 4°C with rotation. Meanwhile, 200 μ l 50% Protein G Agarose beads were centrifuged at 4 150 x g for 2 minutes and supernatant removed. These beads were washed three times with 100 μ l IP Dilution Buffer, centrifuging at 4 150 x g for 2 minutes. Protein G Agarose beads were resuspended using 500 μ l of the chromatin/IgG solution and then transferred back to the falcon tube. Samples were incubated at 4°C overnight on rotation at 15 rpm. Next day, chromatin was centrifuged at 1 690 x g for 2 minutes at 4°C, then supernatant transferred to a new 15 ml falcon tube, centrifuged at 1 690 x g for 2 minutes at 4°C to remove any non-specific chromatin bound IgG beads.

4.5.6 Immunoprecipitation of chromatin

Pre-cleared chromatin was aliquoted for each immunoprecipitation condition, 900 μ l per condition. The following conditions were used: No antibody, IgG, HMGN3 Antibody, Anti-acetyl-Histone H3 (Lys14) Antibody (Upstate). A no chromatin control was included in which 900 μ l IP Dilution Buffer were used in place of chromatin, while a 180 μ l chromatin sample controlled for the starting material was delegated input. The desired quantity of antibody was added to the appropriate 900 μ l chromatin and left at 4°C for 2 hours on rotation (4.8 μ g IgG, 4.8 μ l H3K14ac, 12 μ g mN3Ab). The input sample was left at 4°C on rotation until the reversal of protein-DNA crosslinks was carried out. Protein-DNA complexes were precipitated with 72 μ l 50% Protein G Agarose beads which had been prepared by centrifuging at 5 150 x g for 2 minutes at 4°C and supernatant removed. After 2 hours, the chromatin/antibody solution was centrifuged at 18

000 x g for 5 minutes at 4°C before transferring supernatant to tube containing the Protein G Agarose beads, mixed and left at 4°C for 2 hours on rotation at 20 rpm. The beads/chromatin were then centrifuged at 5 150 x g for 2 minutes at 4°C. Having removed the supernatant a series of washes followed. First, 500 µl IP Wash I was added, vortexed briefly, centrifuged 2 minutes at 5 150 x g at 4°C then supernatant removed. Another 500 µl IP Wash I added, mixed then transferred to a new 15ml falcon tube, centrifuged at 5 150 x g for 2 minutes, 4°C and supernatant removed. Beads were next washed with 500 µl High Salt Buffer, centrifuged at 5 150 x g, 2 min, 4°C, supernatant removed then washed with 500 µl IP Wash II, centrifuged as previous and supernatant removed. A subsequent two washes of the beads with 500 µl TE Buffer, centrifuged as before, then removal of supernatant completed the washes. To elute chromatin, 150 µl Elution Buffer was added to the beads, vortexed briefly, centrifuged 2 minutes at 5 150 x g at room temperature. Supernatant was collected before a second elution with a fresh 150 µl Elution Buffer was conducted in the same way, with the two elutes being combined. Protein-DNA crosslinks were removed by adding 20 µl 5 M NaCl (10.8 µl for input), along with 1µl RNase (1 mg/ml) (to degrade RNA and aid purification of DNA), and left at 65°C for 4 hours. Then 3 µl (20 mg/ml) proteinase K was added and left at 45°C overnight.

4.5.7 Recovery of DNA

DNA was recovered from samples using Phenol:Chloroform. TE (120 µl) was added to input sample, before 300 µl Phenol:Chloroform was added to all samples, vortexed, centrifuged at 18 000 x g for 5 minutes and top layer transferred to a new tube. To each sample was added 30 µl 3 M sodium acetate (pH 5.2) and 0.5 µl glycogen (10 mg/ml), mixed then 750 µl 100% ethanol added, mixed and subsequently incubated at -20°C for at least 30 minutes. Samples were centrifuged at 18 000 x g for 15 minutes at 4°C, supernatant removed then 800 µl 70% ethanol added, mixed by inversion and centrifuged at 18 000 x g for 5 minutes. Supernatant was removed and samples left to air dry at room temperature. DNA was resuspended in 200 µl RNase-DNase-free water at 50°C for 5 minutes. DNA was then used immediately for QRT-PCR or stored at -20°C.

4.5.8 QRT-PCR of DNA

Immunoprecipitated DNA was quantified using QRT-PCR. QRT-PCR reactions were carried out in a total volume of 25 μ l containing 5 μ l of immunoprecipitated DNA, 12.5 μ l FastStart SYBR Green Master Mix (Roche), 300 or 900 nM forward and reverse primers and prepared to a final volume of 25 μ l with DNase-RNase free water. The primers used for QRT-PCR are listed in Table 1. Samples were loaded to a 0.2 ml semi-skirted 96-well PCR plate (colour natural) using an automatic dispensing pipette, sealed then centrifuged at 125 x g for 1 minute before being loaded into a Stratagene MX3000P PCR machine. The PCR reactions were carried out using the following conditions: an activation step of 1 cycle 10 minutes 95°C, an amplification phase consisting of 40 cycles of denaturation 10 minutes 95°C, annealing 1 minute 55°C, extension 30 seconds 72°C, then 1 cycle consisting of 1 minute 95°C, 30 seconds 55°C, 30 seconds 95°C to generate a dissociation curve. Data was analysed using the Stratagene MX3000P v3.20 Build 340, Schema 74 software. The reference dye Rox was used to normalise the minor differences present between samples well, such as differences in the volume of QRT-PCR reaction or transparency of Sealing Film. Results were calculated as fold enrichment of immunoprecipitated DNA over input, rather than the $\Delta\Delta C_t$ method, as the reference gene, β -Globin, was found to bind HMGN3.

4.6 Dual-luciferase reporter assay system

This assay requires the simultaneous expression and measurement of *Photinus pyralis* (firefly) and *Renilla reniformis* (*Renilla*) activity from the one sample. The firefly reporter is correlated with the effect TNF α has on Dox-induced and uninduced cells, while *Renilla* normalises for variability between samples that may arise due to differences in cell viability, transfection efficiency or differences in pipetting volumes.

4.6.1 Transfection

T23 cells were plated onto a white 96-well, clear bottomed plate at a density of 2.22×10^4 cells per well and grown in 50 μ l of medium overnight as described in Section 4.3.3. Cells were transfected the following day with a combination of

the plasmids listed in Table 3. For each reaction, 1-40 ng of the appropriate plasmids and 25 µl of Opti-MEM (1x) were added to a tube, mixed and left at RT for 5 minutes. Meanwhile, for each reaction 0.26 µl of Lipofectamin™ 2000 Transfection Reagent (Invitrogen) and 25 µl Opti-MEM (1x) were added to a 15 ml falcon tube, mixed and left at RT for 5 minutes. After 5 minutes, the appropriate volume of transfection mix from the falcon tube was added to the tubes containing plasmids, mixed then left at RT for 20 minutes. During this time, cells were washed with PBS, before replacing media with 50 µl DMEM + GlutaMAX-1 (1x) containing no antibiotics. Dox (0.1 µl of 2 µg/ml) was added to the appropriate wells. Finally, 50 µl of the transfection mix containing plasmids was added to the correct wells and the cells returned to the incubator for 24 hours. After the incubation period, cells were washed with PBS before 50 µl DMEM + GlutaMAX-1 (1x) supplemented with 0.1% serum medium was added. Another 50 µl of 0.1% serum medium containing 2x concentrate of 0.05 µg/µl TNFα and or 2x concentrate of 2 µg/ml Dox were added to the appropriate wells, while uninduced cells received a further 50 µl of 0.1% serum medium only. Cells were returned to the incubator for 24 hours to allow protein synthesis of luciferase.

4.6.2 Dual luciferase assay

The luciferase assay was performed using the Dual-Luciferase® Reporter Assay System (Promega). Having prepared the kit reagents as per manufacturers guidelines and primed the luminometer injectors, cells were removed from the incubator and washed with 100 µl PBS before 40 µl PLB (1x) was added to the cells and left at RT on a rocker for 15 minutes. The 96-well clear-bottomed plate was read using a luminometer. Raw data was exported to MS excel, normalised and analysed.

Primer	Sequence 5'-3'
ATCA-0002F	GGCATGCCTTGCCTGAGTAA
ATCA-0054R	GCTCGTTCATCCCTGCTGA
HTCA-3598F	ATGCCAATGCCAGAAAGCTT
HTCA-3648R	GCTCATAGGAGGCCCTCAAGA
ITCA-4265F	ACTAGGCTCAGCGGGCAAAC
ITCA-4345R	TGGAATGATCTTGTAACCCCAAGA
KTCA-4850F	GGCCATTGGGTATGAGTTCCT
KTCA-4900R	CATGGAGAAGAGGTGGGAGTCC
OTCA-7070F	CCCAGCAGGATGGTGTGAGT
OTCA-7121R	TGCCTCTGCCTCATCTAGCAC
TTCA-9775F	GGATGTGAAGAGACTGCAGCC
TTCA-9831R	GCTTGGCAACTTCCCACAAG
mBmaj-174F	GTCATCACCGAAGCCTGATT
mBmaj-22R	TGTCTGTTTCTGGGGTTGTG
bmaj-3970F	CACCGAAGCCTGATTCCGTA
bmaj-4020R	GAGCAGATTGGCCCTTACCAG
Glyt 14727F	GGTACTCATGGAACATGGTGCTG
Glyt 14727R	CCAGATGTGGTTGGATACCCA
Glyt 18231F	GTGCCCAGGAATTGTACTAATCAATA
Glyt 18231R	CCACACCCTCTCTCCCATAG

Table 1 Genomic QRT-PCR primers

Primer	Sequence 5'-3'
GAPDH-419F	GATGCCCCCATGTTTGTGAT
GAPDH-568R	GGTCATGAGCCCTTCCACAAT
mN3-103F	GTTCCACCAAACCGGAGTCT
mN3-212R	CCAGCTTCCTGCTTTTCTTCC
tca3-163F	CTATTGGAGACCGTAAGCATGCT
tca3-113R	CGTGTGGATACAGGATGTTGACA

Table 2 cDNA QRT-PCR primers

Plasmid	Function
pNF- κ B-Luc	NF- κ B Reporter
pFC-MEKK	Positive NF- κ B Control
pCIS-CK	Negative NF- κ B Control
pFR-Luc	Fusion Reporter
pFA2-CHOP	Fusion <i>Trans</i> -Activator
pFC-dbd	Negative Fusion Control
pFC-MEK3	Positive Fusion Control
pRL-TK	<i>Renilla</i> Reporter

Table 3 Plasmids

5 Results

5.1 *mCCL1* expression

The aim of the first experiment was to confirm the previous finding that HMGN3 reduces the level of TNF α -induced *mCCL1* expression. This work was done in T23 cells, which are Tet-On MEFs that induce HMGN3 expression upon exposure to Dox (Section 4.3.2). The cells were grown in the presence or absence of Dox in 10% serum medium and then arrested by reducing the serum concentration to 0.1%. Growth arrest served to ensure gene expression be at a minimal level prior to TNF α induction. After 16-24 hours, 0.05 $\mu\text{g}/\mu\text{l}$ TNF α was added together with fresh 10% serum medium and left to incubate for 4-6 hours, before harvesting for RNA.

The effect of adding fresh 10% serum medium on *mCCL1* expression was unknown. In order to determine whether the fresh serum was important for the effect of HMGN3, this experiment was repeated as before, but with an additional experiment where fresh 0.1% serum medium was added at the time of adding TNF α . RNA was extracted 4 hours later and analysed by quantitative RT-PCR (QRT-PCR).

The data shows induction of HMGN3 with Dox is not affected by the serum concentration in either TNF α -induced or uninduced cells, with a ~28 and ~24-fold increase in expression, respectively (Figure 5A). However, HMGN3 expression is slightly lower in cells induced with TNF α .

The data shows that 10% serum medium enhanced the level of TNF α -induced *mCCL1* expression in HMGN3 induced cells from 17 to 48-fold and in HMGN3 uninduced cells from 33 to 64-fold (Figure 5B). This data also shows HMGN3 reduces the level of TNF α -induced *mCCL1* expression by around 50% in low serum conditions, from 33 to 17-fold, and is reduced in 10% serum medium from 64 to 48-fold (Figure 5B), thus, confirming the previous finding.

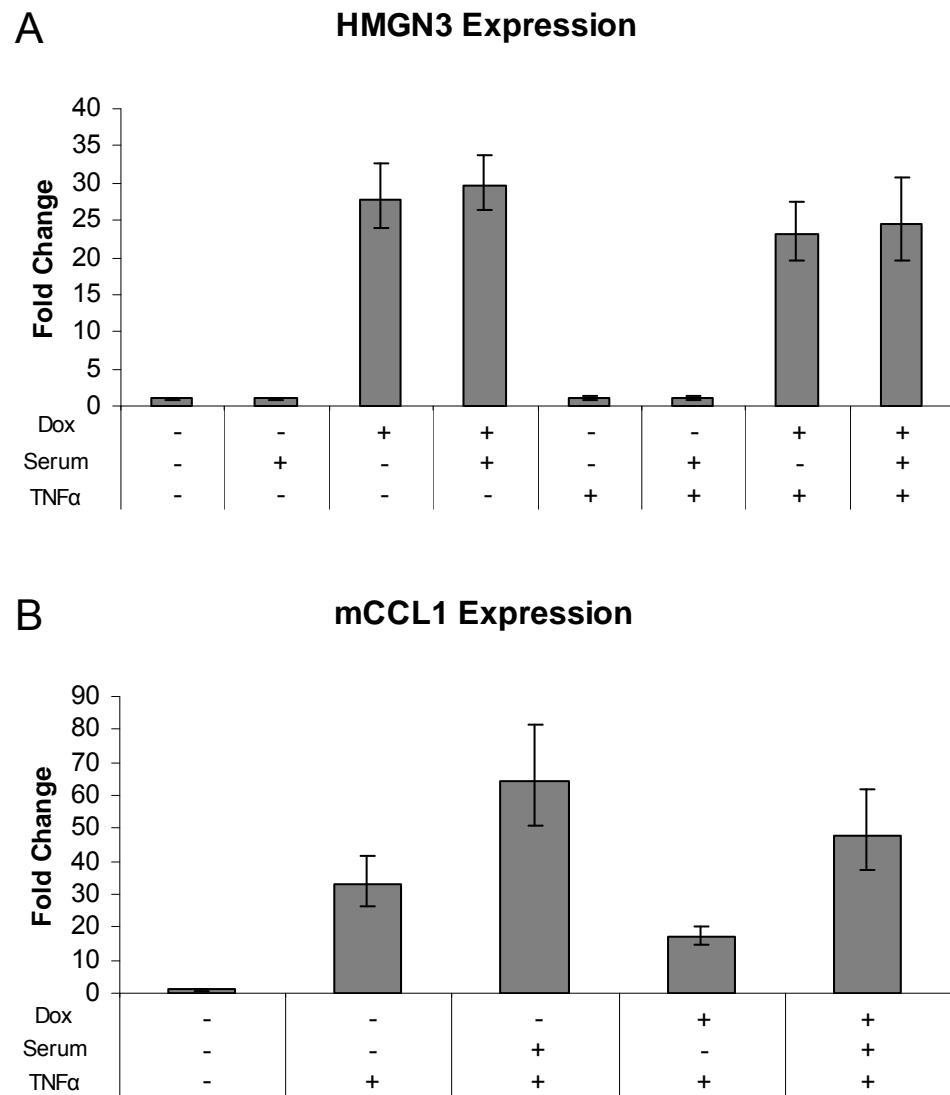


Figure 5 HMGN3 reduces the level of TNF α -induced mCCL1 expression.

Whole cell RNA was extracted from T23 MEFs, and HMGN3 and mCCL1 expression analysed by QRT-PCR. Sample data was normalised to GAPDH and to the corresponding gene expression in uninduced T23 MEFs. Primer sequences shown in Table 2. Cells were treated with or without 0.05 $\mu\text{g}/\mu\text{l}$ TNF α , 2 $\mu\text{g}/\text{ml}$ Dox, as described in Materials and Methods Section 4.3.3. TNF α and Dox was mixed with either fresh 0.1% (-) or 10% (+) serum medium prior to being added to cells, then left to incubate for 4 hours. (A) HMGN3 RNA expression data. (B) mCCL1 RNA expression data. Data presented were obtained from three experimental replicates, with triplicate QRT-PCR reactions for each. Data points represent means \pm SEM.

5.2 *TNF α -induced mCCL1 expression*

Although previous results had shown *mCCL1* mRNA expression increased with 4-6 hours of TNF α incubation, the optimal incubation time was unknown. To determine this, RNA was extracted every 30 minutes from TNF α -induced T23 cells, over a period of 6 hours. QRT-PCR analysis of cDNA synthesised from the extracted RNA revealed mCCL1 expression was greatest after 2 hours incubation with TNF α (Figure 6). Elevated levels of mCCL1 expression were detectable within 30 minutes of adding TNF α , peaking at 2 hours with approximately 70-fold increase over uninduced cells. mCCL1 mRNA expression remained elevated for the duration of the 6 hour period, being around 40-fold higher at 4 hours and 13-fold at 6 hours.

From the data obtained in Sections 5.1 and 5.2, all future experiments required T23 cells to be arrested in 0.1% serum medium, typically for 24 hours, then receive fresh 0.1% serum medium with or without 0.05 $\mu\text{g}/\mu\text{l}$ TNF α , with or without 2 $\mu\text{g}/\text{ml}$ Dox, before being harvested 2 hours after TNF α induction.

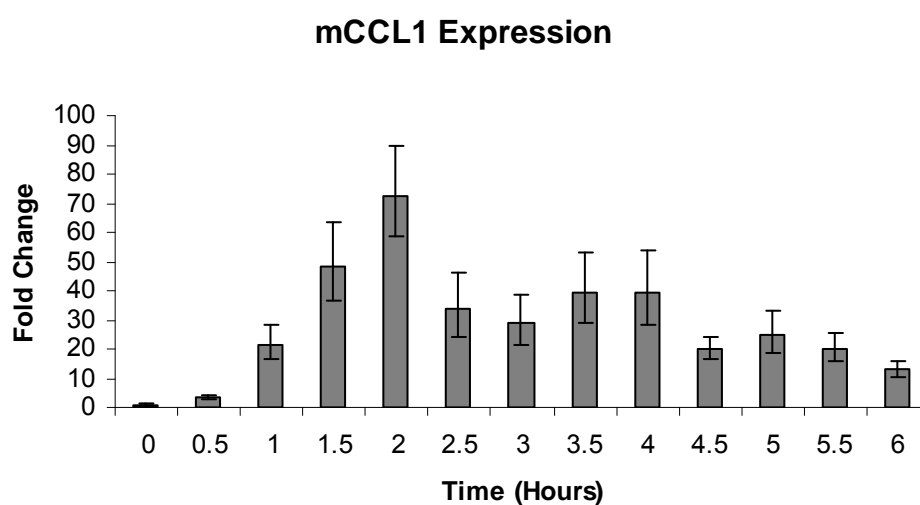


Figure 6 Time course of TNF α -induced mCCL1 expression.

Whole cell RNA was extracted from MEFs at the specified time points and mCCL1 expression analysed by QRT-PCR. Gene expression values were normalised to GAPDH and to mCCL1 expression of cells at time point zero hours. Primer sequences shown in Table 2. Cells were treated as described in Materials and Methods Section 4.3.3 with 0.05 $\mu\text{g}/\mu\text{l}$ TNF α , 10% serum medium, but without inducing for HMGN3 (no Dox added). Data presented were obtained from three experimental replicates, with triplicate or duplicate QRT-PCR reactions for each. Data points represent means \pm SEM.

5.3 *ChIP for HMGN3 at mCCL1 gene*

The HMGN family are nucleosome-binding proteins. Previous studies identified HMGN3 target genes, including Glyt1a (West *et al.*, 2004b). HMGN3 up-regulates Glyt1a expression (in Hepa-1 cells) and is recruited to the Glyt1a gene in cells.

ChIP assays were performed to show whether HMGN3 binds directly to the mCCL1 gene in T23 MEFs. Cells grown in the presence or absence of Dox were incubated with or without 0.05 $\mu\text{g}/\mu\text{l}$ TNF α for 2 hours and then fixed with formaldehyde as described in Material and Methods Section 4.5. Chromatin fragments were prepared by sonication, then immunoprecipitation for HMGN3 or H3K14ac carried out. Chromatin fragment lengths in the range of 400-1200bp were sought and detected from agarose gel electrophoresis. Both the sonication conditions and antibody concentration for immunoprecipitation were optimised (data not shown). The level of HMGN3 associated with the mCCL1 gene was assayed using QRT-PCR primer sets corresponding to the mCCL1 gene and the flanking genomic regions. Exact primer sequence locations are illustrated in detail in Appendix I. Primer concentrations were optimised and dilution series carried out to show linearity (data not shown). The ChIP results presented reflect the level of HMGN3 bound at the region encompassed by the primer set at any given time due to the constant association and disassociation of HMGN proteins on chromatin.

5.3.1 *HMGN3 binds mCCL1 chromatin*

Cells grown in the absence of both Dox and TNF α indicate HMGN3 binds to the mCCL1 gene, with enrichment ranging from 2.5 to 10-fold (Figure 7A, blue line, open circles; see also Figure 7E), while cells grown in the presence of Dox have much higher enrichment levels, ranging from 41 to 91-fold (Figure 7A, red line, open squares; Figure 7C).

TNF α treatment of Dox cells reduced HMGN3 binding to a fairly constant level of 50-fold enrichment across the locus (Figure 7A, red line, closed squares; Figure 7D). Similarly, TNF α treatment of no Dox cells reduced HMGN3 binding from 2.5-10 fold to approximately 3-fold across the locus (Figure 7A, blue line, closed circles; Figure 7F). IgG controls are also shown (Figure 7, panels C-G).

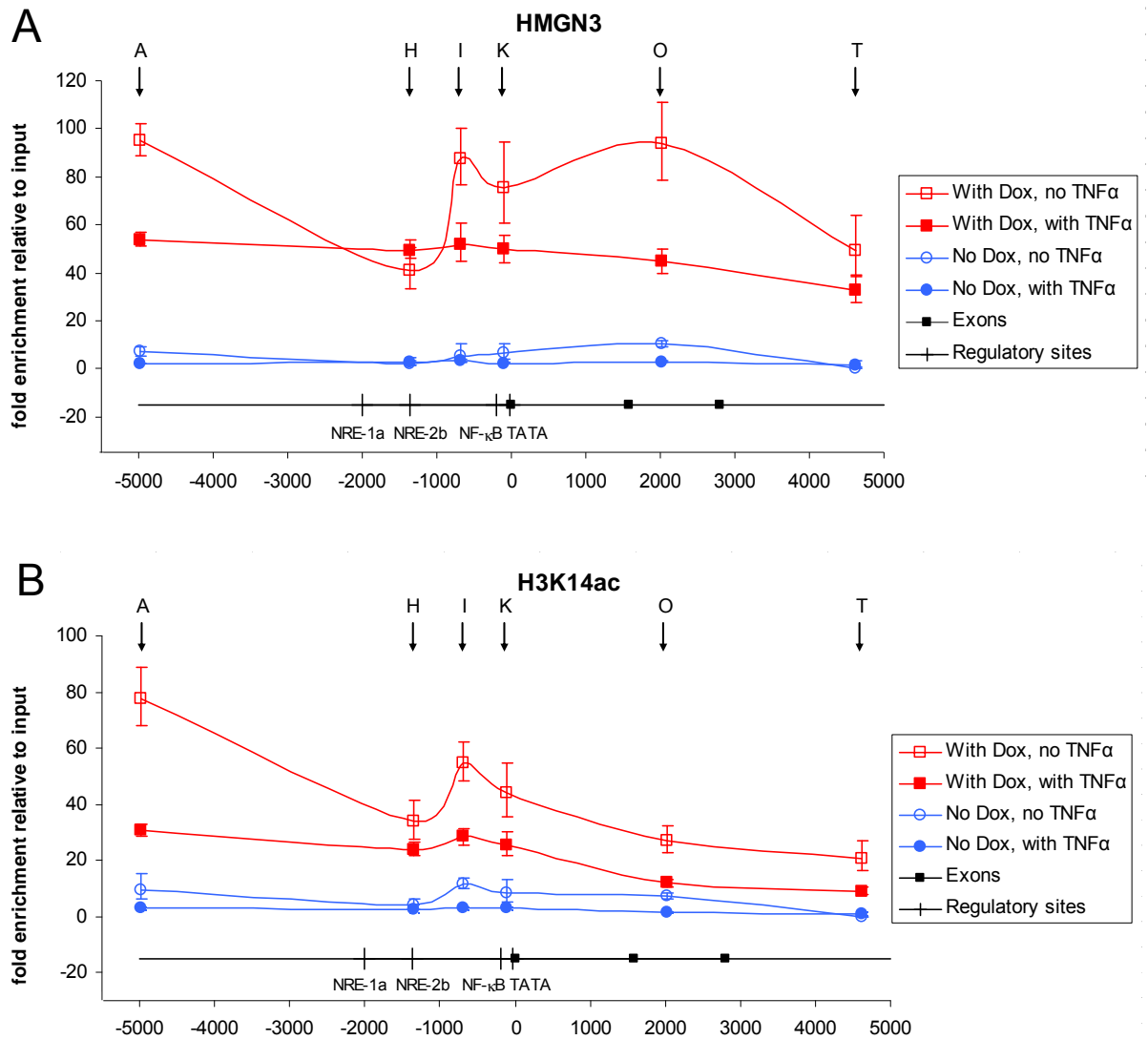


Figure 7 ChIP assays for HMGN3 binding and H3K14ac across the mCCL1 gene in T23 MEFs.

Panels A and B collate HMGN3 and H3K14 data, respectively, from panels C-F for ease of interpretation. Panels A and B show the mCCL1 gene and exons in black from 5' to 3', with numbering relative to the transcription start site (+1). Regulatory sites NRE-1a (-1999), NRE-2b (-1362), NF- κ B (-194) and TATA box (-29) are indicated. mCCL1 primer sequences indicated by A, H, I, K, O, and T can be found in Table 1 and Appendix I. Location of primers in panels C-F are shown to scale in panel G. Cells were incubated with 0.05 μ g/ μ l TNF α for 2 hours and then fixed with formaldehyde as described in Material and Methods Section 4.5. Enrichment levels are relative to input DNA. Data presented were obtained from triplicate QRT-PCR assays. Data points represent means \pm SD.

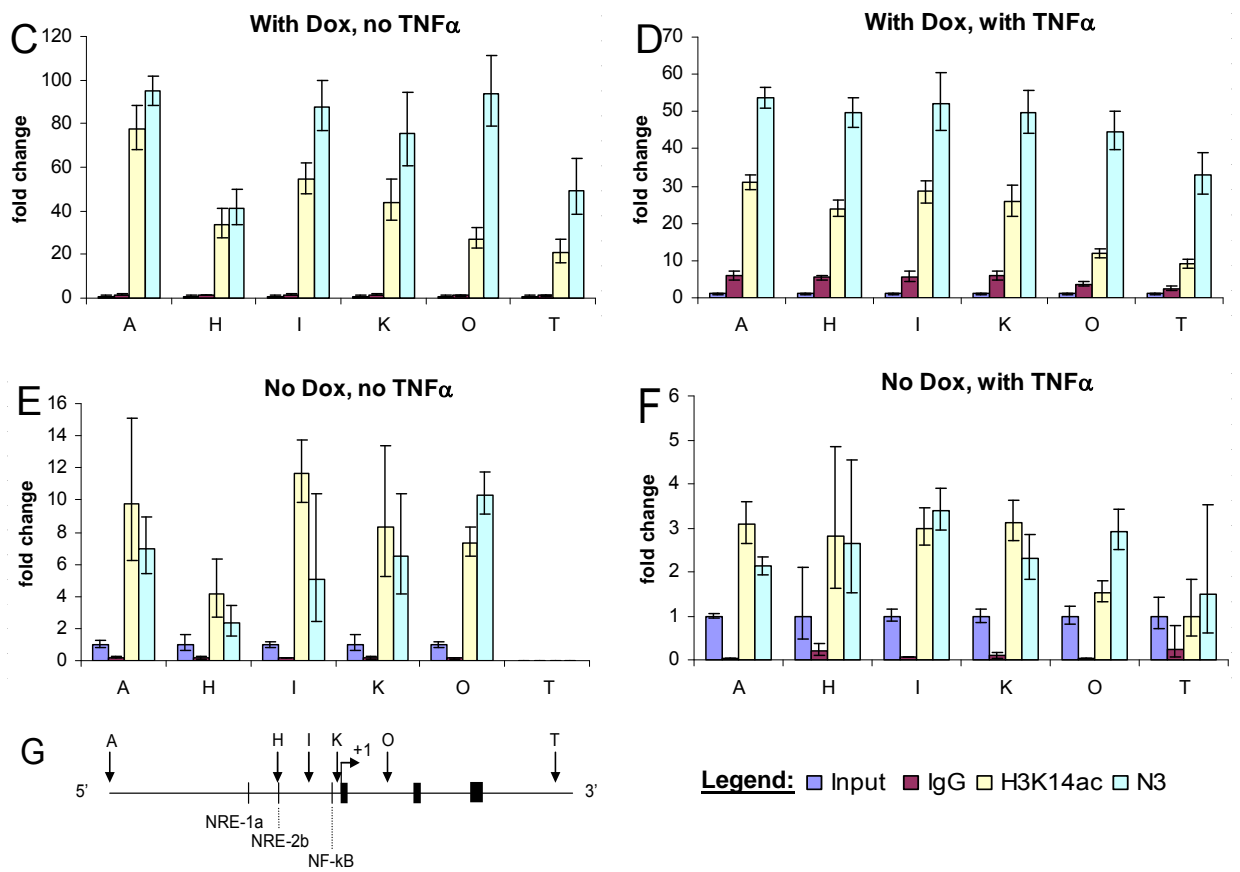


Figure 7 (continued)

5.4 CHIP for H3K14ac at mCCL1 gene

Having shown HMGN3 binds at the mCCL1 gene, ChIP assays were performed for the histone modification H3K14ac. This epigenetic mark is characteristic of active transcription start sites and promoter regions (Poholok *et al.*, 2005; Roh *et al.*, 2005). In addition, evidence suggests upon TNF α stimulation, IKK α mediates acetylation of H3K14 and phosphorylation of H3S10, stimulating transcription.

5.4.1 H3K14ac across mCCL1 gene

Induction of HMGN3 with Dox causes an increase in H3K14ac across the mCCL1 gene, from 4-12 fold to 20-80 fold enrichment (Figure 7B, compare blue line, open circles with the red line, open squares; Figure 7E and 7C). Noticeably, H3K14ac peaks at -4973 and -690bp from the TSS. Interestingly, TNF α treatment of Dox cells reduced the level of H3K14 acetylation by around 50% (Figure 7B, compare red line, open squares to red line, closed squares). In particular, a marked reduction in acetylation occurred at the previous peaks at -4973 and -690bp, almost abolishing these acetyl peaks. Similarly, following TNF α induction of no Dox cells, H3K14ac levels decreased from 4-12 fold to 1-3 fold (Figure 7B, compare blue line open circles with blue line, closed circles; Figure 7E and 7F). Ideally, for each condition (ie. with or without Dox, with or without TNF α) parallel experiments with Histone H3 core antibodies should be carried out to normalise for potential differences in nucleosome density between induced and uninduced cells, and to account for any differences in antibody affinity.

5.5 Summary - H3K14ac tracks with HMGN3

These data show HMGN3 levels partially track with H3K14ac levels. Dox-induced cells have high levels of H3K14ac at the mCCL1 gene, in particular at peaks -4973 and -690bp which correlate with HMGN3 peaks. Upon TNF α stimulation, the levels of H3K14ac at the mCCL1 gene fall, and this fall correlates with reduced HMGN3 binding. This suggests HMGN3 recruits H3K14ac activity, similar to that of HMGN1 which was found to elevate H3K14ac levels by enhancing HAT activity (Lim *et al.*, 2005).

To investigate whether HMGN3 modulates acetylation of H3K14 at other gene loci, CHIP was performed at two locations on the Glyt1 gene. Importantly, Glyt1 is not transcribed in T23 cells. Cells grown in the absence of Dox indicate HMGN3 binds to the Glyt1 gene (Figure 8). In the presence of Dox, enrichment of HMGN3 at the Glyt1 gene is increased from 22 to 57-fold, however, H3K14ac levels remain steady at around 20-fold (Figure 8), showing HMGN3-enhanced acetylation is not universal.

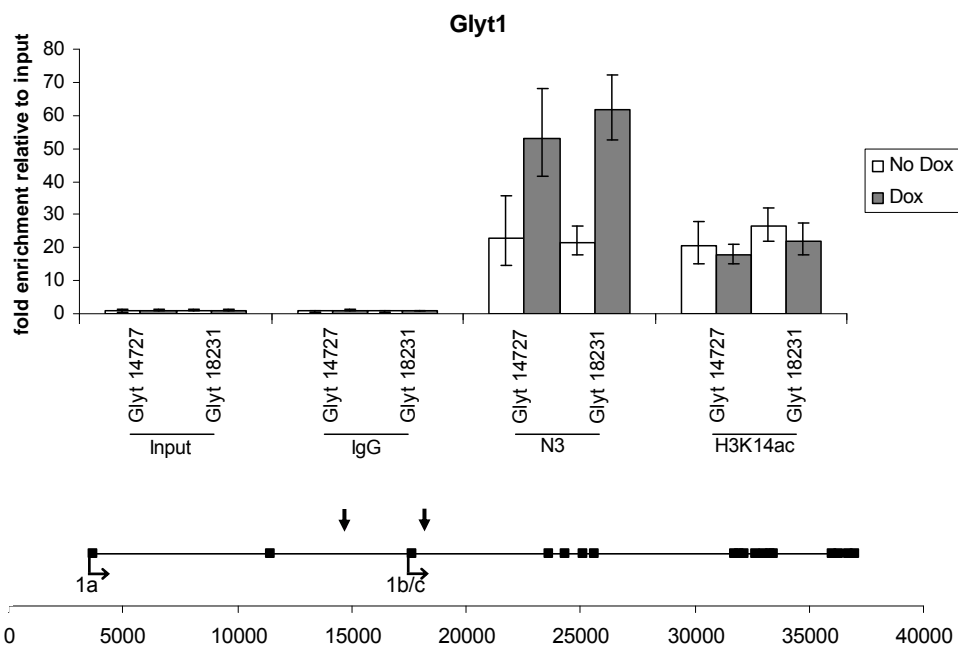


Figure 8 ChIP assays for HMGN3 or H3K14ac enrichment at the Glyt1 gene in T23 MEFs.

Cells were grown then fixed with formaldehyde as described in Material and Methods Section 4.5. Enrichment levels are relative to input DNA and were determined by QRT-PCR. Glyt1 primer sequences shown in Table 1. The positions of the two Glyt1 primer sets are shown beneath the graph as vertical arrows. The two Glyt1 promoters (1a and 1b/c) and exons are shown. Data presented were obtained from triplicate assays. Data points represent means±SD.

5.6 Role of HMGN3 in NF- κ B and p38 pathways

TNF α induces the NF- κ B and p38 (Beyaert et al, 1996) signalling pathways. The mCCL1 gene contains an inducible NF- κ B element at position -194 to -185, relative to the TSS. To investigate whether HMGN3 affects either the NF- κ B or p38 signalling pathways, Dual-Luciferase Reporter Assays were performed as described in Section 4.6. NF- κ B or p38 reporter plasmids were incubated with cells for 24 hours to allow transfection. Activation of the NF- κ B and p38 pathways leads to expression of the luciferase reporter gene which can be quantified.

5.6.1 NF- κ B plasmids

The NF- κ B reporter plasmid (pNF- κ B-Luc) contains direct repeats of the NF- κ B transcription factor recognition sequence. This controls expression of the *cis*-reporter *Photinus pyralis* (firefly) luciferase gene which can be measured using a luminometer. The NF- κ B negative control plasmid (pCIS-CK) contains the luciferase reporter gene but lacks the NF- κ B DNA elements, while the positive control plasmid (pFC-MEKK) contains a kinase that is constitutively expressed and activates transcription of the luciferase gene.

5.6.2 p38 plasmids

The p38 system requires transfection of two plasmids. The reporter plasmid (pFR-Luc) contains five tandem repeats of the yeast GAL4 binding sites that control expression of the *cis*-reporter *Photinus pyralis* (firefly) luciferase gene. The Fusion *Trans*-Activator plasmid (pFA2-CHOP) expresses a fusion protein containing the activation domain of CHOP fused with the GAL4 DNA binding domain. CHOP is a transcription factor that is activated following p38 activation, which occurs following TNF α stimulation. The Fusion *Trans*-Activator negative control plasmid (pFC-dbd) contains the DNA-binding domain of yeast GAL4 but lacks the activation domain, while the positive control plasmid (pFC-MEK3) constitutively expresses the MEK3 kinase (Mitogen-activated protein kinase 3) which phosphorylates and activates p38.

5.6.3 Optimisation of plasmid ratios

The transfection conditions for the plasmids were first optimised. pRL-TK, which constitutively expresses *Renilla* (*Renilla reniformis*) luciferase, was used as a transfection control. T23 cells were transfected with the plasmids then treated with TNF α . The Dual-Luciferase[®] Reporter Assay (Promega) was used to quantify expression of both firefly and *Renilla* luciferase. An optimal pNF- κ B-Luc to pRL-TK ratio was determined by altering both the quantity and ratio of the plasmids (Figure 9A). An optimal pNF- κ B-Luc to pRL-TK ratio of 20:1, using 0.04 μ g pNF- κ B-Luc to 0.002 μ g pRL-TK, was chosen as this yielded the greatest induction of luciferase expression (Figure 9A, indicated by black arrow). A cell density of 2.2×10^4 per well and an incubation time of 24 hours yielded the optimal increase in luciferase expression (Figure 9A, indicated by black arrow).

To maintain continuity, the same cell density, incubation time and quantity of pRL-TK was used for p38 transfections. The optimal quantity of pFR-Luc to pFA2-CHOP was found to be 0.04 μ g to 1 ng (Figure 9B, indicated by black arrow).

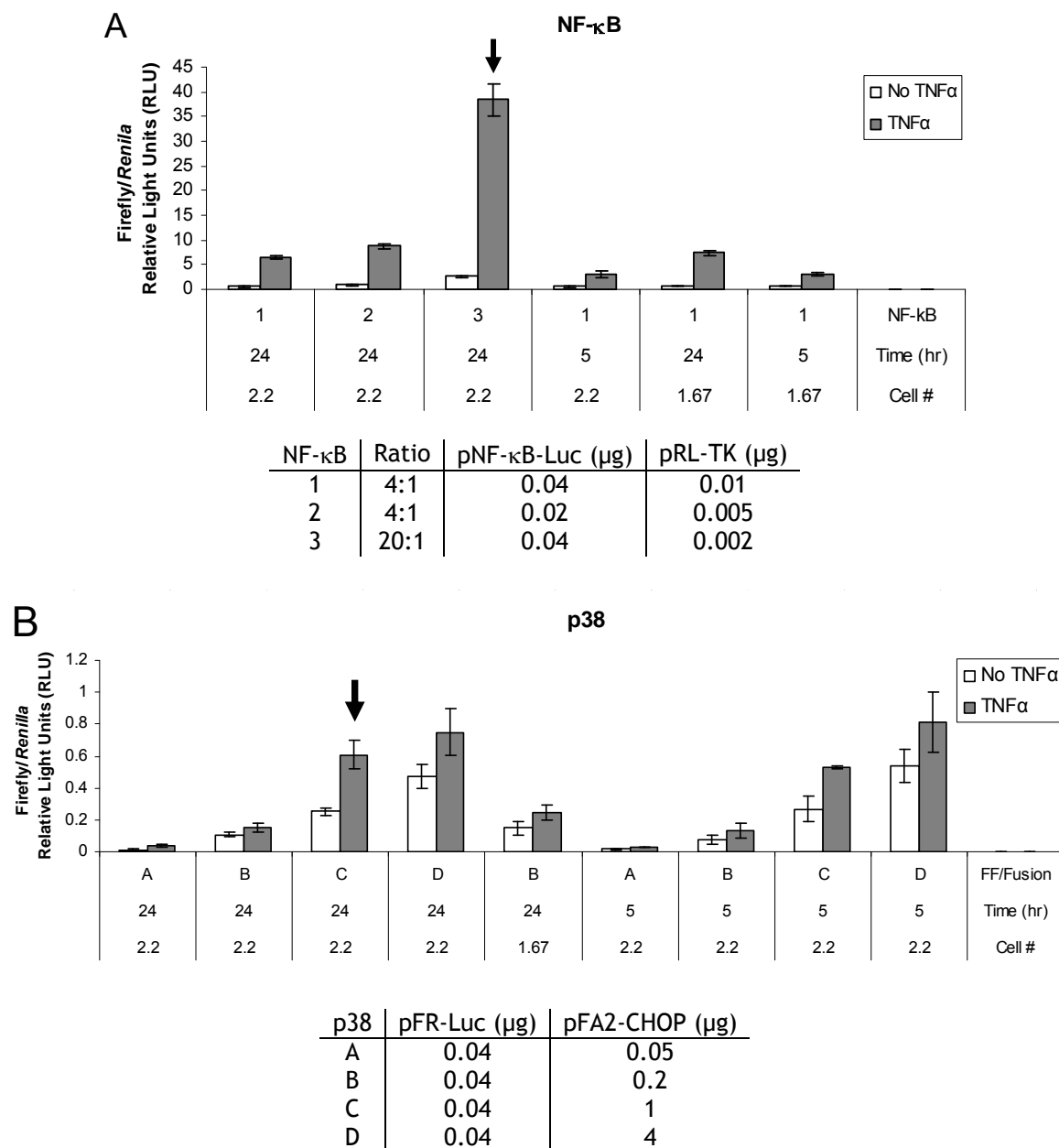


Figure 9 Optimisation of plasmid ratios and quantities.

The Dual Luciferase Assay was carried out as described in Material and Methods Section 4.6. The plasmid ratios and quantities are shown in the tables below the graphs. Cell densities of either 2.2×10^4 cells or 1.67×10^4 cells were investigated. Plasmids were incubated with T23 cells for 24 hours to allow transfection. Cells were treated with $0.05 \mu\text{g}/\mu\text{l}$ of $\text{TNF}\alpha$ for either 24 hours or 5 hours, then all assays were analysed using the luminometer. Data points represent mean and standard deviation of quadruplicate assays. Subsequent conditions used are indicated by black arrows.

5.7 *NF- κ B* luciferase results

To identify whether HMGN3 affects the NF- κ B signalling pathway, T23 cells grown in the presence or absence of Dox were incubated with plasmids for 24 hours (Materials and Methods Section 4.6.1). Cells were then incubated with 0.05 μ g/ μ l TNF α for 24 hours to allow luciferase expression, before luciferase expression was quantified using the Dual-Luciferase[®] Reporter Assay.

Following TNF α treatment, NF- κ B-dependent luciferase expression increased 6-fold in cells uninduced for HMGN3, and 4-fold in HMGN3 induced cells (Figure 10A). Similar results were obtained from repeating this experiment twice more. A Student's t-test was performed to determine whether the difference is statistically significant. For each experiment, HMGN3 was shown not to have a statistical significant role in affecting the NF- κ B signalling pathway, although a near statistical significance ($p = 0.0571$) was obtained for the data presented (Figure 10A).

5.8 *p38* luciferase results

Similarly, to identify whether HMGN3 affects the p38 signalling pathway, luciferase assays were carried out as described previously.

Following TNF α treatment, both HMGN3 induced and uninduced cells had a 1.6-fold increase in p38-dependent luciferase expression (Figure 10B). Repeating this experiment twice more produced similar results. Performing a Student's t-test showed no statistically significant difference in luciferase expression. It is worth noting luciferase expression was low in cells transfected with the positive control pFC-MEK3 (1.4-1.8-fold, Figure 10B), and low in the optimisation assays too (Figure 9B). This suggests the p38 signalling pathway is not very active in T23 MEF cells and is not highly induced following TNF α stimulation.

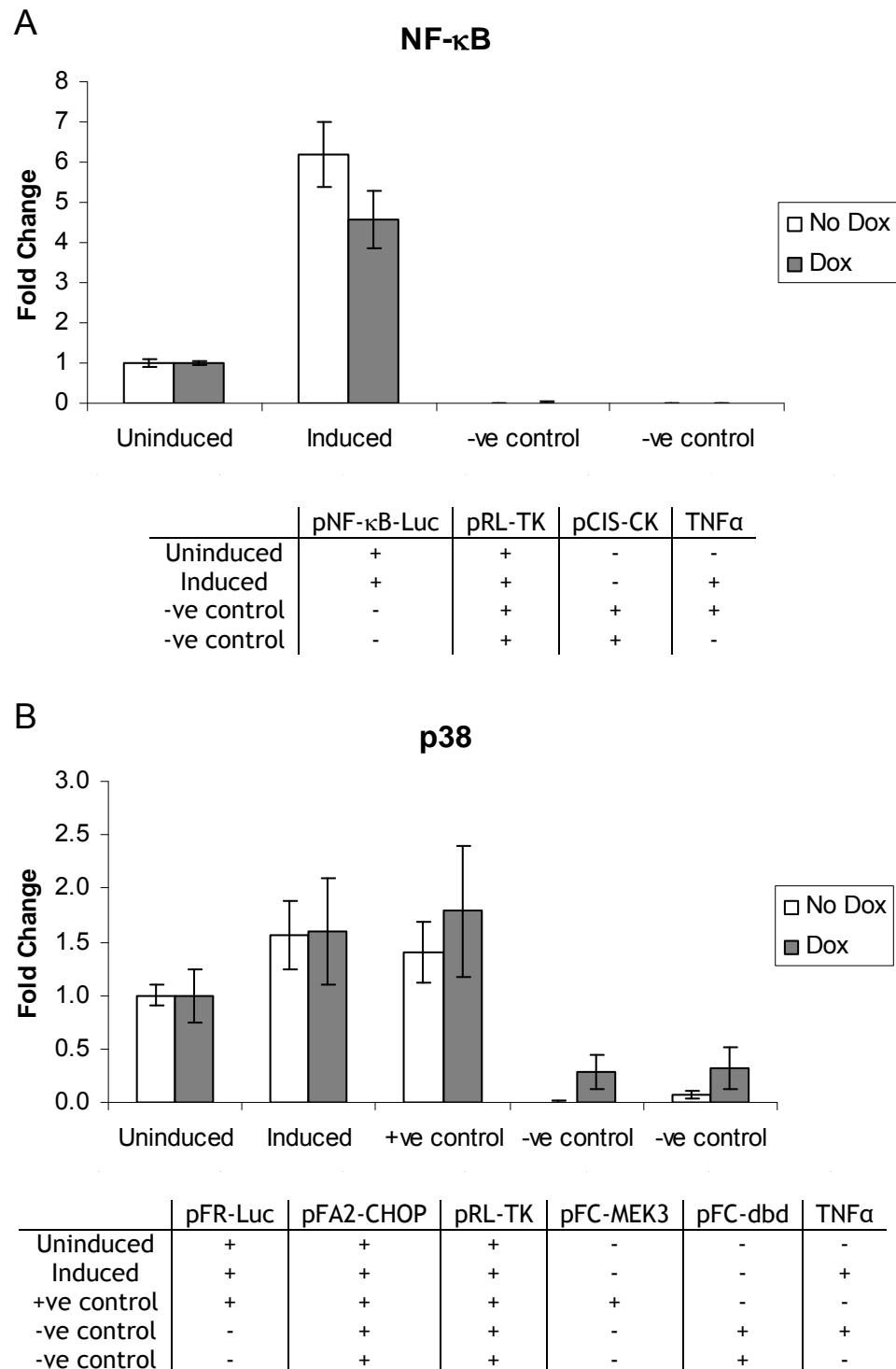


Figure 10 Luciferase assays study the effect of HMG3 on NF- κ B and p38 signalling.

Luciferase assays were carried out as described in Material and Methods Section 4.6. Cells plated at a density of 2.2×10^4 cells were incubated with the appropriate plasmids for 24 hours to allow transfection. Cells were then incubated with or without $0.05 \mu\text{g}/\mu\text{l}$ TNF α for 24 hours. Results are normalised to cells uninduced with TNF α . (A) A pNF- κ B-Luc to pRL-TK ratio of 20:1 was used, using $0.04 \mu\text{g}$ pNF- κ B-Luc to $0.002 \mu\text{g}$ pRL-TK. In addition, the positive control plasmid (pFC-MEKK) indicated the reporter system was working (data not shown). (B) $0.04 \mu\text{g}$ pFR-Luc and 1 ng of pFA2-CHOP were used, along with $0.002 \mu\text{g}$ pRL-TK. The data shown are representative of three independent experiments. The data represents mean and standard deviation of triplicate assays. (A) ($p=0.0571$). (B) ($p=0.9223$)

5.9 Summary of HMGN3 in NF- κ B and p38 signalling

In conclusion, following TNF α stimulation the NF- κ B pathway is induced in T23 MEF cells, however, HMGN3 does not have a statistically significant role in altering NF- κ B signalling. TNF α stimulation leads to a modest increase in p38 signalling, however, HMGN3 does not significantly alter p38 signalling.

6 Discussion

HMGN proteins bind to nucleosomes and modulate chromatin structure. These proteins reduce the compactness of the chromatin fibre and enhance accessibility to nucleosomes overall, but they also alter the accessibility of residues on the nucleosomes they bind (Ding *et al.*, 1997). HMGN proteins modulate histone modifications, such that HMGN1 and HMGN2 enhance acetylation of Histone H3 lysine 14, while HMGN1 inhibits phosphorylation of Histone H3 serine 10 and serine 28 (Lim *et al.*, 2004; Ueda *et al.*, 2006). As a result, HMGN proteins are important in modulating transcription and replication (Bustin, 2001). Although HMGN proteins reduce the compactness of chromatin and improve access of transcriptional activators, this will also improve access for transcriptional repressors.

HMGN3 is unique amongst the HMGN family as two splice variants are produced from the HMGN3 transcript (West *et al.*, 2001). Unlike HMGN3a, HMGN3b lacks most of the CHUD, although the functional consequences of this require further investigation. Both HMGN3a and HMGN3b bind to the promoter region of the *Glyt1* gene and up-regulate expression of *Glyt1* (West *et al.*, 2004b).

Previously, HMGN3 was shown to reduce the level of TNF α -induced expression of the murine chemokine gene CCL1. The aim of this investigation was to determine the role of HMGN3 in regulating gene expression by studying the mouse cytokine gene CCL1. The mRNA data confirms HMGN3 reduces the level of TNF α -induced mCCL1 expression. The ChIP results presented here show HMGN3 binds across the mCCL1 gene, and HMGN3 binding correlates with H3K14ac. Peaks in H3K14ac coincide with peaks of HMGN3 binding at the mCCL1 gene. TNF α stimulation leads to decreased HMGN3 binding and this correlates with reduced H3K14ac at the mCCL1 gene. This is reminiscent of a previous study that showed HMGN1 inhibits anisomycin-stimulated fosB transcription (Lim *et al.*, 2004). Taken together, this shows that HMGN3 fine-tunes TNF α -induced gene expression, and suggests it may be necessary to remove HMGN3 from the mCCL1 gene in order to get efficient transcription.

The mCCL1 gene contains an inducible NF- κ B element. TNF α stimulates the NF- κ B and p38 signalling pathways. An important step in NF- κ B activation is

phosphorylation of I κ B by IKK, as this allows NF- κ B to translocate from the cytoplasm into the nucleus where it can bind to the NF- κ B element and activate transcription (Figure 4). IKK consists of two kinases, IKK α and IKK β , and a regulatory subunit IKK γ . IKK β and IKK γ are critical for cytokine induced I κ B degradation. IKK α , however, can directly phosphorylate H3S10 and enhance acetylation of H3K14 at NF- κ B regulated promoters (Anest *et al.*, 2003; Yamamoto *et al.*, 2003). Phosphorylation of H3S10 seems to be important for the subsequent acetylation of H3K14 by CBP (Yamamoto *et al.*, 2004). p38 also induces phosphorylation of H3S10, which may improve access for NF- κ B to bind the NF- κ B site (Saccani *et al.*, 2002).

To gain insight into whether HMGN3 affects mCCL1 gene expression through either the NF- κ B or the p38 signalling pathways, luciferase assays were performed. HMGN3 was shown not to have a statistically significant role in either the NF- κ B or p38 signalling pathways, although HMGN3 was shown statistically to have a near significant role ($p = 0.0571$) in the NF- κ B signalling pathway.

As just mentioned, HMGN1 mediates transcription from the *fosB* gene, whereby following stimulation, HMGN1 bound at the *fosB* gene is phosphorylated by MSK1, reducing HMGN1 binding to chromatin, and enhancing the accessibility of MSKs to phosphorylate H3S10 (Lim *et al.*, 2004). HMGN3 may participate by a similar mechanism. HMGN3 binds mCCL1 chromatin, enhances H3K14ac as shown by ChIP, and may inhibit TNF α -induced H3S10 phosphorylation, possibly mediated by IKK α , inhibiting mCCL1 expression as shown by RNA data. However, following TNF α stimulation, HMGN3 may become phosphorylated, possibly by IKK α or MSK1, reducing HMGN3 binding to chromatin as suggested by the ChIP results, enhancing the accessibility of H3S10 phosphorylation, possibly by IKK α (Figure 11). The reduced binding of HMGN3 may be necessary for efficient expression of the mCCL1 gene.

Enhanced H3K14ac mediated by HMGN3 is gene specific, as H3K14ac levels were unaffected by increased HMGN3 enrichment at the Glyt1 gene.

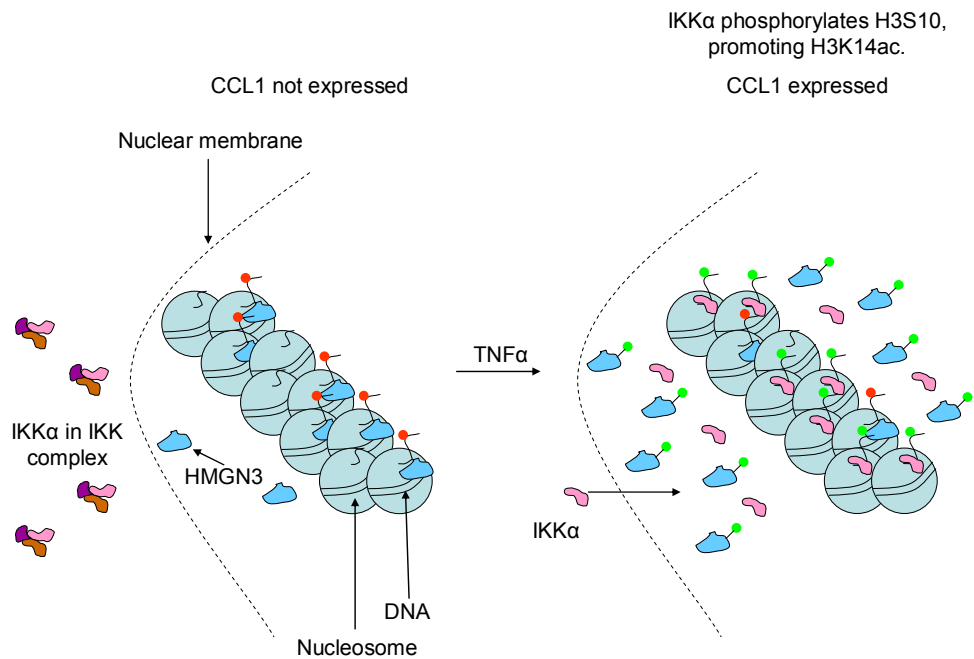


Figure 11 Reduced HMGN3 binding to nucleosomes enhances H3S10 phosphorylation.

Prior to TNF α stimulation, IKK α is in the IKK complex and HMGN3 binds to nucleosomes at the mCCL1 gene, inhibiting transcription. TNF α stimulation leads to the activation of the NF- κ B pathway, IKK α translocates into the nucleus, HMGN3 becomes phosphorylated weakening its interaction with the nucleosome, allowing IKK α to phosphorylate H3S10 and initiate transcription. As the magnitude of the stimulus recedes, nuclear IKK α levels decrease, HMGN3 is de-phosphorylated favouring re-association with the nucleosome, eventually restoring transcriptional repression. Acetylated, red circles; Phosphorylated, green circles.

6.1 Further work

To investigate whether H3S10 becomes phosphorylated following TNF α stimulation, Western analysis of cells incubated with or without TNF α should be carried out. If H3S10 becomes phosphorylated, whether this modification occurs at the mCCL1 gene can be investigated by CHIP assays, using chromatin from cells incubated with or without TNF α . As IKK α can phosphorylate H3S10 treating cells with IKK α inhibitors would reveal whether this kinase is responsible for H3S10ph following TNF α stimulation.

HMGN proteins regulate gene expression by mediating access of histone modifying enzymes to their nucleosomal targets. These enzymes not only target histone tails, but also HMGN proteins. p300, a histone acetyltransferase, also acetylates HMGN1 and HMGN2 at multiple sites, weakening HMGN binding to the nucleosome, thus improving access of p300 to induce histone changes (Bergel *et al.*, 2000). PCAF, also a histone acetyltransferase, can also acetylate HMGN2 which reduces the affinity of HMGN2 for nucleosomes (Herrera *et al.*, 1999). MSK1 and RSK2, two kinases known to phosphorylate H3S10 and H3S28, also efficiently phosphorylate HMGN1 which precedes histone phosphorylation (Lim *et al.*, 2004). As IKK α can phosphorylate H3S10, IKK α is a key candidate as the potential kinase for phosphorylation of HMGN3. Western analysis of HMGN3 will show whether HMGN3 becomes phosphorylated following TNF α stimulation, and identify the residues targeted.

If, following TNF α stimulation H3S10 becomes phosphorylated, CHIP assays or Western analysis for H3S10ph will show whether HMGN3 inhibits H3S10ph as suggested here. Further investigation will show whether TNF α induces other histone modifications at the mCCL1 gene, such as H3K4me3 and H3K4me, which are found at active promoters and enhancers respectively (Heintzmann *et al.*, 2007). In contrast, H3K27me negatively regulates transcription (Martin and Zhang, 2005). CHIP assays would then show whether these modifications are affected by HMGN3.

In addition to H3S10ph, H3K14ac and H3S28ph, HMGN proteins also affect H3K9me, H3K9ac (Lim *et al.*, 2004) and H2AS1ph (Postnikov *et al.*, 2006). Further investigation will show whether combinations of histone modifications

are induced by $\text{TNF}\alpha$, such as H3S10p with H3K9ac or H3S10ph with H3K14ac, and whether these are affected by HMGN3. As a result, knowledge of the relationship between HMGN3 and gene expression will be improved.

Further ChIP assays will establish a higher resolution map of HMGN3 binding, and for the associated histone modification H3K14ac across the mCCL1 gene. From the results obtained, higher levels of H3K14 acetylation occur 5 kb upstream of the mCCL1 TSS. High levels of H3K14ac are found within the 700 bp region extending upstream from the TSS. This region has been shown to enhance mCCL1 expression (Oh and Metcalfe, 1994). A lower level of H3K14ac is observed at primer set H which lies at the negative regulatory element-2b. Further investigation is required to see if these trends continue across the regions associated with transcriptional enhancement (1342bp upstream from the TSS) and repression (between 1342 to 2057bp upstream of TSS).

In summary, HMGN3 binds mCCL1 chromatin and enhances H3K14ac. HMGN3 may hinder the accessibility of kinases, such as $\text{IKK}\alpha$, to H3S10 in nucleosomes, and thus inhibit induction of transcription. On this basis, HMGN3 functions similarly to HMGN1 at the *fosB* gene, where HMGN1 enhances H3K14ac and hinders phosphorylation of H3S10, reducing gene induction in response to anisomycin.

7 References

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8 Appendix I

Nucleotide sequence for mCCL1 gene, including flanking regions. Numbers to left of sequence are relative to TSS. Location of regulatory sites and exons shown to right of sequence and highlighted in colour in sequence. Location of forward and reverse primers “A, H, I, K, O and T” are shown to right of sequence and underlined in sequence. 1st number in bracket after primer indicates primer name in Table 1, 2nd number corresponds to primer location relative to TSS in the sequence shown.

Mus musculus (lab mouse) (Build 37.1)

Chromosome 11 Minus Strand From: 81988137 To: 81998296
Contig: NT_096135.5 Start: 47493015 Stop:47503174 Strand: Minus

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-5000 gggccttcaa gactacgcat gatggaaggg atgccttgctc ctgagtaaac catggtgaca Primer "A"(0002F, -4973)
-4940 atcagcaggg atgaacgagc cttgtcaaca taatcttgctg taacccgact tgggcctctc Primer "A"(0054R, -4921)
-4880 cctattcatt tttagagccc gcctccacac ttgtgcttga cctcactgct cccattttcc
-4820 ttgagaactc tgccaggcat tgttctgagc atattcctcc aacgccatac caagcctata
-4760 agcagcgggt attcacatat ccatttagag atgagctcag agctgaagca ttcaaaggag
-4700 cctggactgg cagcctacag gttggcccag aacctgctca aaaccactac catggtagct
-4640 ccacacattc ttcccctctg ggtacaccct gcttgcattc ttccaggcct aggtgaaata
-4580 ccacactgct acctaaggag cccttagcct ctagaccata cactaatgac agcctgtgct
-4520 cagctggttg tttgtgggtg ctctttcccc taaatctggc cctggagggc agatctgagc
-4460 tatgatccct tctcttgctc cagtaccag gcacgaactg ctaaacagcg ggtctctgga
-4400 agatgcctac agagagaata gaggagcatt ccactaaacc atagctgtcc cagggacccc
-4340 aactttaata tgttgtccac acagaaattg caagatttcc agcagtggaa ctctgtataa
-4280 acagatgtag tttcagaacc cagttactta ttctgggttc caaataagta actggtcacc
-4220 tgtgtgctca ctatcacctc ctgactgccc actaagggtt gaggcctcag tgggtacaaa
-4160 cttctttctt cttatttcca gtagtactg catcccacat acagatggca aagagaggtc
-4100 tgactgggag agaaagagtt ctgctctcta gagctcctgg agaggggtggg acatggaacc
-4040 ctaggggaacc aaaacctgca gttgctgggt ctctccagga gctcgggggtg gcacagcctg
-3980 tgcagagttg gtcattgtgaa gccaggggaa ggttctgagc aggagaagca gaaggcctga
-3920 catctgaaga gaaggacttc agtgagcagg gccgtcactt cacactttgt aagtgcggcc
-3840 aagacatttg tactttcacc ctgagcacag cgggctggcc tttcaggctc agaagcactt
-3780 gaaagaacca gtgatggtca ctattgtgac aacctctgcc tgcttctatt gtagtgagag

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-3720 acacttccgt gtcctttgtg ctgtaactaa gcagtagaca ggaaagtctc ctgtaaaaga
-3660 acaaagcagc taaaaaacca caggaacagg aagtaaagaa gccagggctc agggctcagg
-3600 attctgtggt gtgtgggtgc tgggcatctt tactgtgaat agacataggc ttacctcagg
-3540 caactgtgtc tggggcagat atcaaggacc cagaaggaca ctgtgaccac atcgtaaag
-3480 acacatcatt aaggacacct ggggtgtctgt agaccaggct agtagcaaaa gagactgggt
-3420 agtagagccc tgtttgggag acagtgacac atcttgggtg caccctgga cctctcagag
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