FACULTE DE BIOLOGIE ET
DE MEDECINE

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Thèse de Doctorat

présentée à la

Faculté de Biologie et de Médecine de
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ISREC
Institut Suisse de Recherche Expérimentale sur le Cancer
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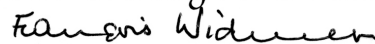le Conseil de Faculté autorise l'impression de la thèse de

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Replicating adenoviruses targeting tumours with constitutive activation of the Wnt signalling pathway

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pour Le Doyen
de la Faculté de Biologie et de Médecine

Prof. François Widmer

Without music, life would be a mistake.

Friedrich Nietzsche

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Abstract

Activation of the Wnt pathway through mutation of the adenomatous polyposis coli and β-catenin genes is a hallmark of colon cancer. These mutations lead to constitutive activation of transcription from promoters containing binding sites for Tcf/LEF transcription factors. Tumour-selective replicating oncolytic viruses are promising agents for cancer therapy. They can in principle spread throughout a tumour mass until all the cancerous cells are killed, and clinical trials have shown that they are safe except at very high doses. Adenoviruses are interesting candidates for virotherapy because their biology is well understood and their small genome can be rapidly mutated. Adenoviruses with Tcf binding sites in the E2 early promoter replicate selectively in cells with an active Wnt pathway. Although these viruses can replicate in a broad panel of colon cancer cell lines, some colorectal cancer cells are only semi-permissive for Tcf-virus replication.

The aim of my thesis was to increase the safety and the efficacy of Tcf-viruses for colon cancer virotherapy. I replaced the endogenous E1A viral promoter by four Tcf binding sites and showed that transcription from the mutant promoter was specifically activated by the Wnt pathway. A virus with Tcf binding sites in the E1A and E4 promoters was more selective for the Wnt pathway than the former Tcf-E2 viruses. Moreover, insertion of Tcf binding sites into all early promoters further increased viral selectivity, but reduced viral activity. I showed that Tcf-dependent transcription was inhibited by the interaction between E1A and p300, but deletion of the p300-binding site of E1A generally led to viral attenuation. In the semi-permissive cell lines, replication of Tcf-viruses remained lower than that of the wild-type virus. The E2 promoter was the most sensitive to the cell type, but I was unable to improve its activity by targeted mutagenesis. To increase the toxicity of the Tcf-E1A/E4 virus, I decided to express a suicide gene, yeast cytosine deaminase (yCD), late during infection. This enzyme converts the prodrug 5-FC to the cytotoxic agent 5-FU. yCD was expressed in a DNA replication-dependent manner and increased viral toxicity in presence of 5-FC. Tcf-E1A and yCD adenoviruses are potentially useful vectors for the treatment of liver metastases from colorectal tumours.

Résumé

Dans la quasi-totalité des cancers du côlon, la voie Wnt est activée par des mutations dans les gènes codant pour APC ou pour la β-caténine. Ces mutations activent de façon constitutive la transcription de promoteurs contenant des sites de liaison pour les facteurs de transcription Tcf/LEF. Les virus réplicatifs spécifiques aux tumeurs sont des agents prometteurs pour la thérapie cancéreuse. En principe, ces vecteurs peuvent se propager dans une masse tumorale jusqu'à destruction de toutes les cellules cancéreuses, et des études cliniques ont démontré que de tels vecteurs n'étaient pas toxiques, sauf à de très hautes doses. Les adénovirus sont des candidats intéressants pour la thérapie virale car leur biologie est bien définie et leur petit génome peut être rapidement modifié. Des adénovirus comportant des sites de liaison à Tcf dans leur promoteur précoce E2 se répliquent sélectivement dans les cellules qui possèdent une voie Wnt active. Ces virus sont capables de se répliquer dans un grand nombre de cellules cancéreuses du côlon, bien que certaines de ces cellules ne soient que semi-permissives pour la réplication des virus Tcf.

Le but de ma thèse était d'augmenter la sécurité et l'efficacité des virus Tcf. Le promoteur viral endogène E1A a été remplacé par quatre sites de liaison à Tcf, ce qui a rendu son activation dépendante de la voie Wnt. Un virus comportant des sites de liaison pour Tcf dans les promoteurs E1A et E4 était plus sélectif pour la voie Wnt que les précédents virus Tcf-E2, et un virus comportant des sites Tcf dans tous les promoteurs précoces était encore plus sélectif, mais moins actif. J'ai montré que l'interaction entre E1A et p300 inhibait la transcription dépendante de Tcf, mais la délétion du domaine concerné dans E1A a eu pour effet d'atténuer les virus. Dans les cellules semi-permissives, la réplication des virus Tcf était toujours plus basse que celle du virus sauvage. J'ai identifié le promoteur E2 comme étant le plus sensible au type cellulaire, mais n'ai pas pu augmenter son activité par mutagenèse. Pour augmenter la toxicité du virus Tcf-E1A/E4, j'ai décidé d'exprimer un gène suicide, la cytosine déaminase (yCD), pendant la phase tardive de l'infection. Cette enzyme transforme la prodrogue 5-FC en l'agent cytotoxique 5-FU. yCD était exprimée après réplication de l'ADN viral et augmentait la toxicité virale en présence de 5-FC. Les virus Tcf-E1A et yCD sont des vecteurs potentiellement utiles pour le traitement des métastases hépatiques de cancers colorectaux.

Part of this work was published in the following papers:

1.   Fuerer C, Iggo R. Adenoviruses with Tcf binding sites in multiple early promoters show enhanced selectivity for tumour cells with constitutive activation of the wnt signalling pathway. *Gene Ther* 2002; **9:** 270-281.

2.   Fuerer C, Iggo R. 5-Fluorocytosine increases the toxicity of Wnt-targeting replicating adenoviruses that express cytosine deaminase as a late gene. *Gene Ther* 2004; **11:** 142-151.

1. Introduction

1.1. Adenoviruses

1.1.1. General description

Adenoviruses (Ad) are mild pathogens that cause respiratory diseases, epidemic conjunctivitis and infantile gastroenteritis. They have an icosahedral capsid and a linear, double-stranded DNA genome. The viral DNA is covalently attached at its 5' termini to the terminal protein (TP) and is associated with core proteins (Figure 1.1). The adenoviral life cycle is divided into two phases, which are separated by the onset of viral DNA replication (Figure 1.2a). Early events comprise the viral entry into the host cell and the subsequent transcription and translation of early genes. Early viral gene products drive the cell into S phase, thus providing an essential environment to sustain viral replication. Late gene products are mainly structural proteins or proteins involved in capsid assembly. To date, more than 50 human adenoviruses have been classified into 6 subgroups, based on their hemagglutination properties and DNA homology. They infect a great variety of post-mitotic cells; even those associated with highly differentiated tissue. In this work, I will focus on human adenovirus type 5 (Ad5), which belongs to the subgroup C of adenoviruses.

1.1.2. Genomic organisation

The adenoviral genome is depicted in Figure 1.2b and contains, adjacent to the coding regions, two inverted terminal repeats (ITRs) and a packaging signal. The ITRs are 103 bp long in Ad5 and are essential for viral DNA replication. The 170 bp packaging signal is located near the left terminus and directs the interaction of viral DNA with the encapsidation proteins. The transcription units are named according to the onset of their transcription during the viral life cycle (early, intermediate, and late).
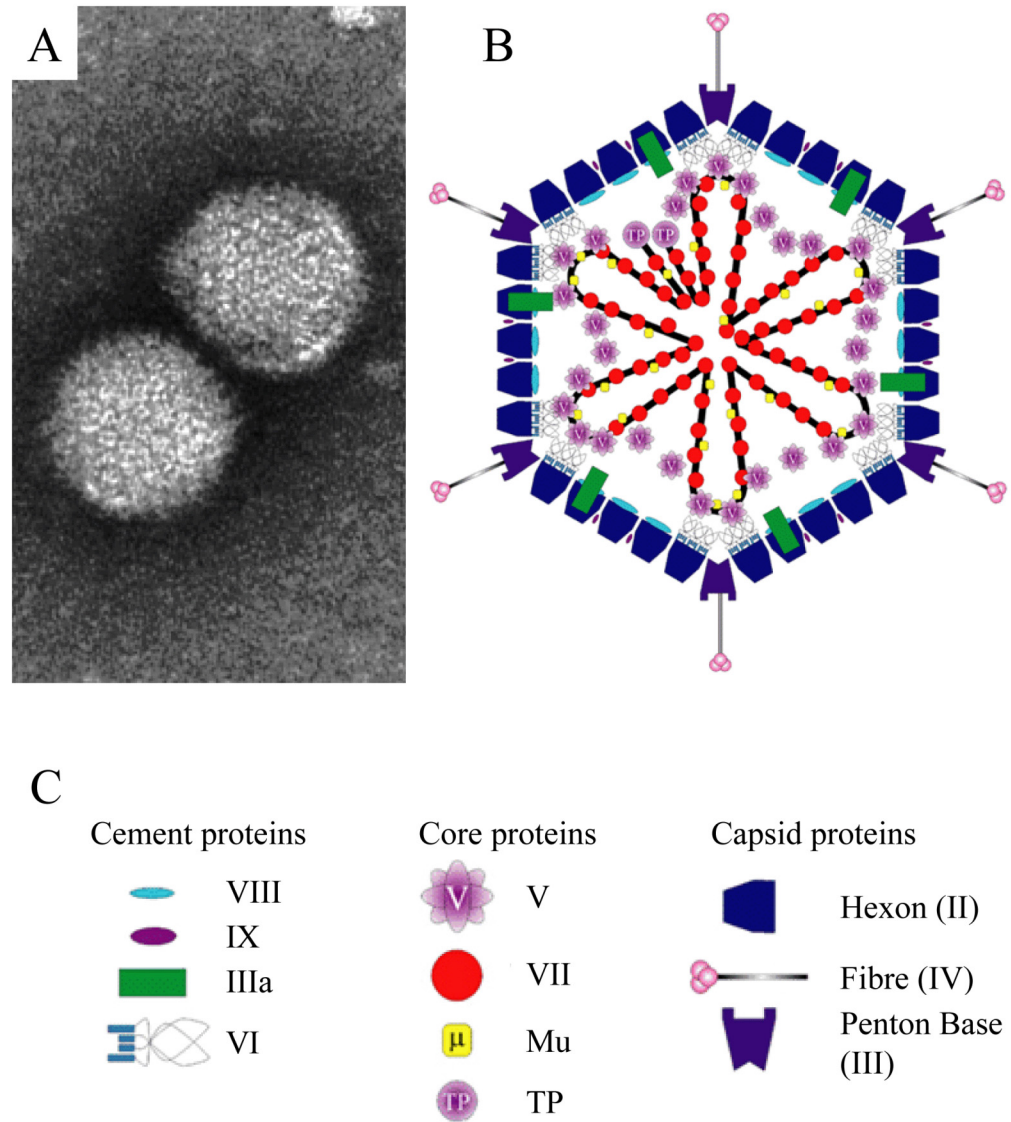
Figure 1.1: (A) Electron microscopy image of adenoviral particles. (A4 virus, courtesy of J. Bamat) (B) Schematic description of an adenoviral particle and (C) Legend.[210]

The viral chromosome carries five early transcription units (E1A, E1B, E2, E3, and E4), two intermediate units (IVa2 and IX) and a major late unit composed of five distinct cassettes (L1, L2, L3, L4, L5). In addition, other late transcripts include those from the late E2 promoter (E2-L) as well as E3 coding sequences that are spliced from the major late promoter (MLP). Finally, the virus encodes two PolIII-dependent RNAs called the virus-associated RNAs (VA RNAs). Their expression is greatly increased during the late phase of infection.

Early transcription   **Late transcription**

Onset of
DNA synthesis

Immediate —————→ Early —————→ Intermediate —————→ Late —————→
–early

E1A   **E1B, E2,**   **IV$_{a2}$, IX**   **L1, L2, L3,**
                        E3, E4   **L4, L5**

(a)

MLTU: **L1**   **L2**   **L3**   **L4**   **L5**
Leaders: 1 2 i 3   x y   z

IX   **VAI,II**   IV
E1B   pVIII –   11.6K
55K   33K
19K   100K –
E1A   PT
9S   pVI   II
12S   pX
13S   V   **E3**
          pVII   12.5K
          III   14.7K
          IIIa   10.4K, 14.5K
          52/55   11.6K
          13.6K   6.7K, gp19K
                                                              11.6K
                                                              6.7K, gp19K

5   10   15   20   25   30   35

                                        E2-L
                                            E2-E
                                    DBP

                                                    Orf1
                                                    Orf2
                                                    Orf3
                                                    Orf4
          pTP
          Pol   Orf6
(b) **IVa2**   **E2B**   **E2A**   Orf6/7
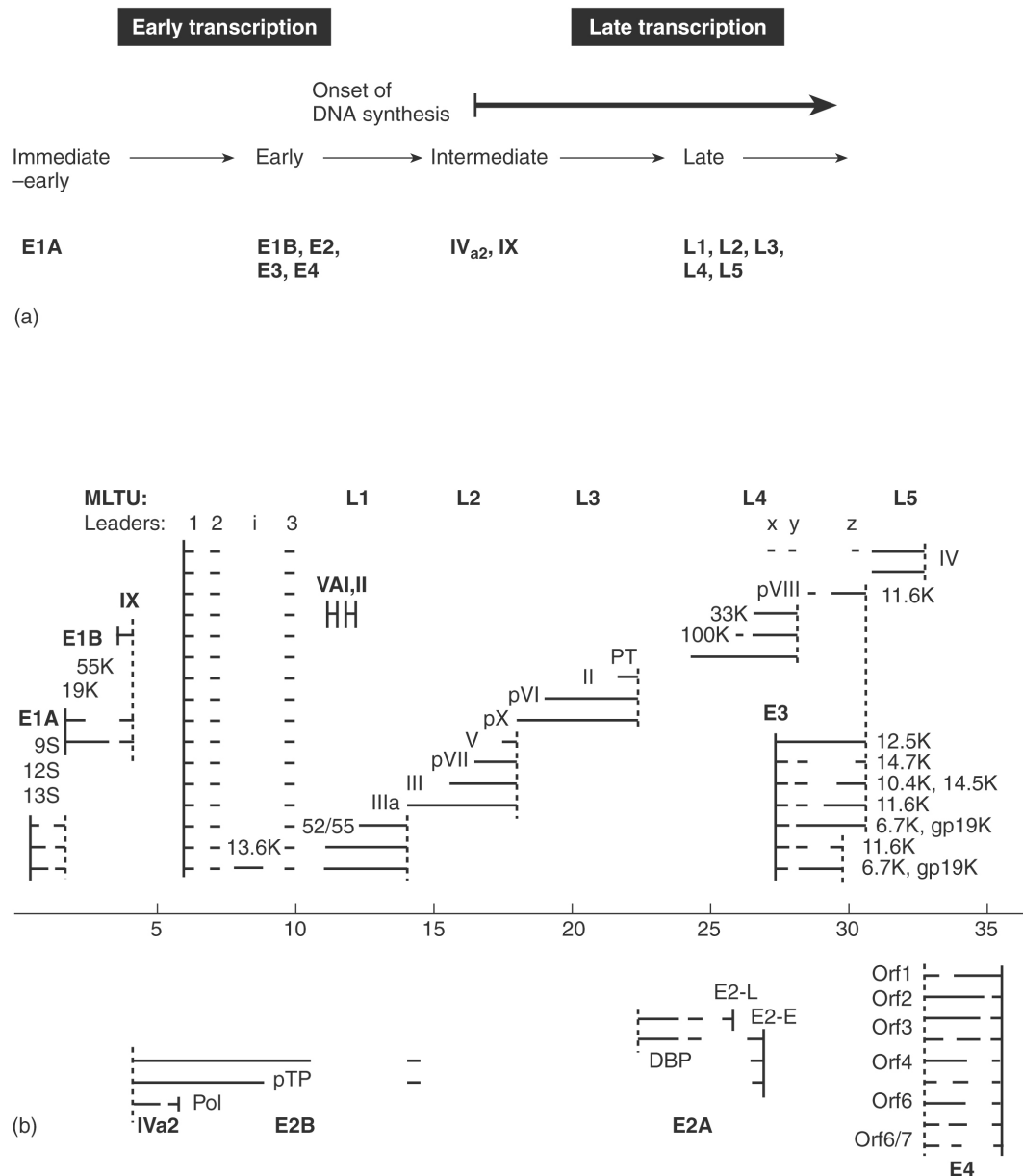   **E4**

Figure 1.2: (A) Adenoviral life cycle. (B) Organisation of the adenoviral genome. The genome size is in kilobases. The genome map is drawn conventionally with the E1A gene at the left end. Both strands are transcribed with the rightward reading strand coding for the E1A, E1B, IX, Major late, VA RNA, and E3 units and the leftward strand coding for the E2, E4, and IVa2 units. [59]

1.1.3. Entry

The attachment of the virus to the cell is mediated by high-affinity interactions between the viral fibre knob and the coxsackie and adenovirus receptor (CAR).[16,19,202] Internalisation is triggered by the interaction between the RGD motif (Arg-Gly-Asp) of the penton protein of the capsid and the cellular integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$.[251] This interaction activates clathrin-

mediated viral endocytosis via α_v integrins, small GTPases, and phosphatidylinositol 3-kinase (PI3K). Release from the early endosomal compartment is driven by the adenoviral protease, reduced pH and probably signalling coming from the integrins. The virus is transported to the nucleus in a microtubule- and dynein/dynactin-dependent pathway. It docks to the nuclear pore complex and get disassembled with the help of nuclear histone H1 and the H1 import factors importin β and importin 7. In addition, the activation of protein kinase C by PI3K can also activate macropinocytosis, a process that provides further internalisation of the virus.[96]

1.1.4. Early transcription units

The early phase of infection has three functions: 1) to drive the cell into S phase, 2) to protect the cell against elicited antiviral defences, 3) to produce viral proteins required for DNA replication. The products of the early transcription units (E1A, E1B, E2, E3, and E4) accomplish these functions and will be described in this chapter.

E1A proteins

The first gene to be expressed from the adenoviral genome is E1A. Transcription of the E1A gene occurs rapidly due to a constitutively active promoter[117] and to the pIX protein of the capsid, which has been shown to transactivate the E1A promoter.[163] Following transcription, alternative splicing of the E1A mRNA leads to production of the 12S and 13S mRNAs, which are translated into the 243R and 289R E1A proteins, respectively. Three smaller E1A mRNA species have been observed later during infection but no role has been assigned to these late peptides so far.[3] The role of the E1A gene products is to force the cells to enter the cell cycle and to transactivate the other early promoters. Comparison of E1A sequences from various serotypes show that E1A proteins have four conserved regions (CR1-CR4).[8] These regions interact with a variety of cellular factors involved in transcription and chromatin remodelling, such as pRb, p300/CBP, ATF, and CtBP (Figure 1.3).

For example, the E2F family of transcription factors regulates several genes that are important for the progression through S phase (such as dihydrofolate reductase, DNA polymerase α, and thymidine kinase). In quiescent cells, the retinoblastoma gene product (pRb) inhibits E2F by direct binding. The binding of E1A to pRb relieves this repression, resulting in activation of E2F-dependent transcription, and thus to promotion of the cell cycle.[45,179]

E1A interacts with the histone acetyltransferases (HATs) p300/CBP[66] and P/CAF[197], but controversial studies indicate that E1A modulates the HAT activity of these proteins either in a positive[2] or negative[40] manner. E1A binds also to a complex containing the SWI2/SNF2-related protein p400 and the cofactor TRRAP,[81] suggesting a role of E1A in the modulation of chromatin remodelling complexes.

The CR4 region of E1A binds to the C-terminal binding protein (CtBP), a corepressor that binds to histone deacetylases. Interaction of CtBP with the E1A C-terminus inhibits CR1-mediated transactivation in *cis*[223] and negatively modulates oncogenic transformation. The E1A-CtBP interaction is decreased by acetylation of E1A by the bound HATs[267] and modulates the effect of E1A on several cellular genes.
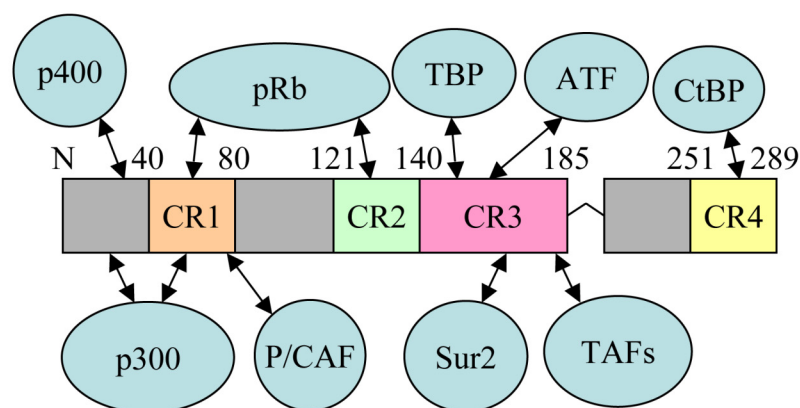

Figure 1.3: Schematic description of the E1A proteins and their binding partners. The CR3 domain is absent in E1A 243R.[210]

The N-terminus of E1A has been shown to interfere with the formation of the TBP-TATA complex,[25] and to be sufficient for binding to p300/CBP[10].

Lastly, the CR3 domain binds to the ATF/CREB family of transcription factors,[44,211] TBP,[89,224] TFIID[88] and Sur2, a subunit of the mammalian Mediator.[27] The transactivating CR3 domain is present in the 289R protein only and probably functions by bridging proteins of the transcription machinery to transcription factors like ATF.

Apart from driving the host cell into S phase, E1A proteins transactivate the other early viral promoters. E1A activates the E2 and E4 promoters by activating E2F and E4F factors, respectively.[179] Similarly, interaction of E1A with ATF results in activation of the E2, E3, and E4 promoters.[150]

E1B proteins

The E1B region encodes the E1B19K and E1B55K proteins, whose major roles are to prevent E1A-induced apoptosis. E1B19K is similar to the anti-apoptotic protein Bcl-2 and prevents procaspase-9 activation.[50,105] E1B55K binds to p53 and can function as a transcriptional repressor.[261] E1B55K, together with E4orf6, also inhibits p53-dependent apoptosis by promoting p53 degradation.[108,195] E1B55K binds to RNA[126] and is involved, together with E4orf6, in the preferential transport and stabilisation of late viral mRNAs.[9,63,191]

E2 proteins

The E2 region codes for proteins of the viral replication machinery and can be subdivided into two regions named E2A and E2B. E2A encodes the single-stranded DNA binding protein (DBP), which is required to stabilise the nascent DNA chain during adenoviral DNA replication.[80] DBP was also shown to activate a variety of viral promoters[42] but other reports showed that DBP has an inhibitory effect on early promoters.[38] E2B encodes the adenoviral polymerase (Pol) and the precursor of the terminal protein (TP). The TP protein is covalently linked to the termini of the viral genome, mediates its association with the proteins of the nuclear matrix and serves as primer during viral replication.[76,190]

E3 proteins

The E3 proteins are involved in the immunomodulation of the host; they play various roles, such as inhibition of antigen presentation, inhibition of ligand-induced cell death, and stabilisation of NF-κB. At least seven proteins are produced from the E3 region.[127,255] (1) E3-gp19k is a transmembrane protein that binds to the MHC class I heavy chain and retains it in the endoplasmic reticulum,[36] thus preventing antigen presentation. (2&3) A heterotrimer of E3-10.4kD and E3-14.5kD (RIDα&β) inhibits TNFα and Fas ligand-induced cell death by internalising their receptors and promoting their degradation in lysosomes.[255] (4) E3-14.7k inhibits TNF-mediated apoptosis by enhancing IKKβ phosphorylation of IKβ, thus stabilising NF-κB.[127] (5&6) The function of E3-12.5kD and E3-6.7kD is not known. (7) The E3-11.6K protein (adenoviral death protein, or ADP) is a glycosylated transmembrane protein required at late stages of infection to accelerate lysis and induce virus release from the host cell.[236] A recent report indicates that this effect might be due to the interaction of E3-11.6K with MAD2B, a spindle assembly checkpoint protein.[262] Although originally described as an early protein, ADP is expressed late during infection from the MLP and must therefore be considered as a late protein.[237] RIDα&β and E3-14.7kD are also detected predominantly late during infection.[111]

E4 proteins

The E4 region encodes at least six distinct polypeptides defined as E4orf1 to E4orf6/7 according to the arrangement of their open reading frame. These proteins have a disparate set of functions, but are mainly involved in the facilitation of viral mRNA metabolism and in the protection of the viral DNA ends.

E4orf3 and E4orf6 are associated with E1B55K.[94,151] E4orf3 reorganises components of nuclear bodies or PML oncogenic domains (PODs) and probably brings E1B55K in close vicinity to these regions. E4orf6 plays a role, with E1B55K, in the preferential cytoplasmic accumulation of viral late mRNAs and in the degradation of p53. In the

cytoplasm, E1B55K and E4orf6 were found in a complex with proteins involved in the ubiquitin/proteasome pathway such as Cullin-5, Rbx1 and Elongins.[108,195] Based on these findings, a model was proposed according to which E1B55K and E4orf6 replace MDM2, the RING-finger protein responsible for targeting p53 to the proteasome pathway. E4orf3 and E4orf6 also enhance viral replication, reorganise the PODs and target the double strand break repair machinery to prevent concatenation of viral genomes. E4orf3 redistributes the double-stranded break repair complex Mre11-Rad50-NBS1 and E4orf6 targets it for degradation.[226] E4orf3 also binds to the DNA-dependent protein kinase (DNA PK) and protects the ends of the viral chromosome from non-homologous end joining.[26] In E4orf3, the inhibition of concatenation can be genetically separated from the enhanced viral replication.[70]

E4orf6/7 transactivates the E2 promoter by binding to free E2F and inducing its dimerisation.[185] The E2F dimer binds with an increased stability to the two inverted E2F-binding sites in the Ad5 E2 early promoter[109] and in the cellular E2F-1 promoter.[215] E4orf6/7 is sufficient to displace pRb and the related protein p107 from E2F complexes and activate E2 expression.[184]

E4orf1 and E4orf6 have partial transforming activities that are able to stimulate E1 transformation. E4orf1 is related to dUTP pyrophosphatases and interacts with PDZ domain-containing cellular proteins (like DLG and MAGI-1).[92] This leads to PDZ protein-dependent activation of PI3K, which contributes to the transforming potential of E4orf1.[77] E4orf4 interacts with PP2A[137] and downregulates AP-1 activity through hypophosphorylation of both c-Fos and E1A.[173] E4orf4 inactivates SR-mediated splicing in L1, probably through dephosphorylation of the SR proteins.[68] Finally, E4orf4 was shown to block yeast and mammalian cells in G2/M.[139]

1.1.5. Replication

Synthesis of new viral DNA is initiated at either terminus of the linear genome. Replication requires the ITRs, where nuclear factor I and III bind to recruit the Ad Pol-pTP complex. Once bound to the ITR, the pTP protein serves as a primer for DNA replication and becomes covalently attached to

the first nucleotide of the nascent strand.[153] Chain elongation is mediated by Ad Pol, DBP and nuclear factor II, a type I topoisomerase.[175] DBP multimerisation results in double-stranded DNA unwinding and enhances Ad Pol processivity.[58] Replication by strand displacement results in a DNA duplex and a molecule of single-stranded DNA. The ITRs of the ssDNA product can anneal intramolecularly and serve as a template for another round of replication.[149]

1.1.6. Late phase and release

Late gene expression occurs after the onset of viral DNA replication. The adenovirus major late transcription unit (MLTU) is differentially spliced and polyadenylated. The resulting mRNAs are classically grouped into five families, termed L1 to L5, based on the utilisation of different polyadenylation sites (Figure 1.2b). This unit is placed under the control of the major late promoter (MLP), whose activation relies on viral DNA replication[238] and on the delayed early viral genes IVa2[162] and pIX.[163] Genes encoded by the MLTU are principally proteins of the capsid and non-structural proteins that have roles in virion assembly.

In the late phase of infection, selective expression of viral proteins is achieved through viral regulation of mRNA export and translation. First, viral mRNAs are selectively exported to the cytoplasm. This selective export depends on E1B-55K, E4orf6 and cellular proteins.[43,85] Further selective expression of viral proteins is achieved by inhibition of the cap-dependent RNA helicase complex eIF4F, which is mediated by the L4 100kD gene product. This helicase is necessary for linear mRNA scanning by the ribosome. Viral mRNAs transcribed from the MLP have a common 5' sequence called the tripartite leader. This sequence helps late mRNAs to bypass the requirement for eIF4F by a mechanism called ribosome shunting.[57,265]

Once the capsid is assembled, the viral DNA packaging signal directs the encapsidation of the viral DNA into the capsid. The L3-encoded protease maturates at least four viral polypeptides to generate the mature VI, VII, VIII, and TP proteins and render the virus infectious. The release of the

progeny virus is facilitated by cytokeratin cleaveage[268] and by the E3 adenovirus death protein (ADP).[236] E4orf4 was shown to increase viral release by inducing apoptosis in a novel, p53-independent manner, in transformed or infected cells.[30]

1.2. Virotherapy

Cancer is the result of abnormal cell proliferation and deregulated apoptosis.[106] Standard chemotherapy and radiotherapy kill cancer by inducing apoptosis but apoptosis resistant clones are likely to emerge during treatment.[114] Tumour-selective replicating oncolytic viruses are promising agents for cancer therapy. They infect, replicate, and kill target cells by various mechanisms. Provided their replication is restricted to cancer cells, they can in principle spread throughout the tumour mass until all the tumour cells are killed. Replicating viruses have a higher therapeutic index than non-replicating viruses because they are selectively amplified in tumour cells, leading to very high local concentrations. As they do not replicate efficiently in normal cells, they have a low associated toxicity and clinical trials have shown that oncolytic agents are safe except at very high doses.

Various strategies and vectors are used to achieve tumour-selectivity. Some viruses such as reoviruses, parvoviruses, Newcastle disease viruses, measles viruses, and vesicular stomatitis viruses, display natural oncotropism, whereas others have to be specifically engineered to become tumour-specific, like adenoviruses, herpes simplex viruses, and vaccinia viruses. In my thesis, I have developed conditionally replicating adenoviruses that target cells with an activated Wnt pathway. Therefore, I will concentrate on the description of adenoviruses as oncolytic vectors. The other vectors mentioned above have been reviewed recently.[114,123,200]

Human adenovirus 5 (Ad5) is a promising candidate virus for tumour therapy because the biology of the virus is well understood and its small genome can be rapidly mutated. The virus can be manufactured relatively easily in large amounts, and clinical trials with attenuated forms have shown that it is well tolerated except at very high doses. Four main types of

modification have been proposed to convert the virus into an effective therapeutic agent. First, tumour-specific replication can be achieved upon deletion of essential viral genes. Second, expression of viral genes can be regulated using tissue- or tumour-specific promoters. Third, mutating proteins of the viral capsid can modify the viral tropism. Finally, the toxicity of the virus can be increased by inserting an unconditional toxin or a prodrug activating enzyme in the viral genome. The following chapters will illustrate briefly these strategies.

1.2.1. Complementary defect approach

Adenoviruses encode proteins that drive infected cells into S phase and anti-apoptotic proteins that prevent premature death of the infected cell. Tumours are defective in controlling cell proliferation and apoptosis. Thus, deletion of viral genes implicated in the control of these pathways will prevent viral replication in normal, quiescent cells, while viral replication will occur in cells that are actively cycling or have a corrupted apoptotic pathway. These two processes are mainly linked to the pRb and p53 pathways, respectively.

The first recombinant adenovirus used to target a cellular defect was the *dl*1520 virus (ONYX-015), which contains a deletion of the E1B55K gene.[22] The rationale was that E1B55K is needed to inhibit p53-dependent apoptosis. In consequence, this virus would be attenuated in normal cells, which have an intact p53 pathway. In most cancer cells, this pathway is defective and should allow the virus to replicate and spread. Although the selectivity of this virus had been questioned,[93,107,208,242] it was used in phase I to III trials before being discontinued. Clinical trials showed that *dl*1520 was safe but elicited few objective responses (14 %).[177] Another study showed that combination of virotherapy and standard chemotherapy led to tumour regression in 63% of evaluable patients with intra-tumoural virus administration.[135] When delivered intra-arterially or intravenously, *dl*1520 failed to demonstrate any significant tumour remission.[100,198]

In addition to its role in inhibition of p53-dependent apoptosis, EIB55K is also implicated in the preferential export of late viral mRNAs.

To circumvent this problem, the Onyx group tested point mutants in the E1B55K protein deficient in down-regulation of p53 only.[219] Two mutants failed to bind to p53 but enabled expression of late viral genes. One virus was able to replicate as efficiently as wild-type in U2OS, a p53-positive cell line, and both viruses showed no striking difference between p53-positive and p53-negative cell lines. This indicates that abolition of p53-binding site in E1B55K is not sufficient to render viral growth p53-dependent. Unfortunately, no data is available on the behaviour of these new E1B55K-mutant viruses in normal cells.

Mutations that abolish the binding of E1A to pRb (ΔRb mutations) have also been reported.[65,84,120] Viruses carrying these mutations are not able to activate the pRb pathway, and thus cannot force cells into S phase to provide an environment suitable for viral replication. Such viruses do not replicate in quiescent, normal cells but they replicate efficiently in tumour cells. Unfortunately, they also replicate in actively cycling normal cells, sometimes even better than wild-type.[120] Overall, their replication is attenuated in normal cells relative to tumour cells, although the wild-type virus was not compared side-by-side in that particular experiment. These viruses are promising agents to target various tumours and have been improved by combining the ΔRb mutation with fibre mutations[147], or with tumour-specific promoters (see below).[132]

Transactivation of differentiation-associated genes by the histone acetyltransferase p300 is inhibited by E1A. Although the basis for the p300 targeting is less clear than for the p53 or pRb targeting, mutations in E1A abolishing p300 binding (Δp300) were introduced either alone or together with the ΔRb mutation.[65,82,120] Viruses harbouring both mutations remain dependent on the cycling state of the cells, similar to the ΔRb single mutants, and the Δp300 mutation was reported to attenuate the virus,[82,120] probably because of the importance of the interaction between E1A and p300 in the viral life cycle. This suggests that the p300-binding site in E1A should be retained in therapeutic viruses.

Viral infection triggers antiviral responses such as the interferon pathway. The double-stranded RNA-activated protein kinase (PKR) is a target of the interferon (IFN) pathway[168] and is activated during infection by

viral dsRNA. Active PKR phosphorylates the α subunit of eIF-2α to inhibit protein synthesis and viral replication.[254] Therefore, bypass of the interferon pathway by therapeutic viruses is essential for viral growth. Virus-associated RNAs I and II (VA RNAs) counteract the IFN response by binding and inhibiting PKR.[134] Downstream effectors of Ras block PKR activation by dsRNA.[174] For that reason, viruses that cannot bypass the PKR pathway can replicate in Ras-transformed cells (defects in the Ras pathway are common in tumour cells). This is the basis of the natural tumour-selectivity of viruses such as measles virus and reovirus.[209,227] To a similar extent, adenoviruses harbouring a deletion in the VAI RNA gene have been shown to target cells with an active Ras pathway.[39] Active Ras can also stabilise specific mRNAs, a feature that was used to target Ras-transformed cells with viruses containing such an RNA-stabilising element in the 3'UTR of the E1A gene.[1]

1.2.2. Tumour-specific promoters

Many kinds of tumour or tissue have been targeted by replacing endogenous viral promoters by tissue- or tumour-specific promoters. For example, prostate targeting was achieved upon replacement of the E1A promoter by the PSA promoter, a prostate-specific cellular promoter.[201] The E1B promoter was further mutated by inserting enhancer and promoter sequences from the prostate-specific kallikrein gene.[264] Alternatively, E1A and E1B promoters were replaced with the prostate-specific rat probasin promoter and the prostate-specific PSA enhancer/promoter, respectively.[263] These viruses lysed efficiently PSA-positive cells and reduced significantly xenografts upon tail vein injection, provided the E3 region was maintained in the constructs.

A broader targeting strategy consists of using the E2F-1 promoter to drive transcription of E1A.[131,240] It has been shown that the E2F-1 promoter is active in tumours and cycling cells *in vitro*. *In vivo*, an E2F-E1A virus was active in cancer cells but not in regenerating liver, suggesting that the levels of "free E2F" was higher in tumours than in cycling cells.[187] The conserved region 2 of E1A is responsible for the transactivation of E2F-

containing promoters. Simultaneous deletion of region 2 and replacement of the E1A promoter by the E2F-1 promoter led to increased selectivity compared to single modifications.[132] Because E4orf6/7 can activate E2F,[185] the E4 region was placed under the control of the E2F-1 promoter, resulting in an even tighter regulation of both E2F-1 promoters present in the virus. Deletion of the open reading frame E4orf7 was reported to have no effect on selectivity, but this experiment was done only in the context of the most selective virus, thus hindering potential changes.[132]

Other targeting strategies rely on selective or over-activation of signalling pathways in a certain subset of cancers. A virus containing the E1A gene under the control of the α-fetoprotein (AFP) promoter was shown to target AFP-positive cells, AFP being expressed in 80% of hepatocellular carcinomas (HCC).[102] Similarly, E1A was placed under the control of the MUC1 promoter to confer breast cancer specificity.[144] To target lung tissue, the surfactant protein B promoter was engineered into already tumour-selective viruses to drive the E4 genes.[64] Insertion of hypoxia-responsive elements in viral promoters leads to hypoxia-dependent regulation of viral genes.[21,124] This is of great interest because the core of the tumour is generally hypoxic. Therefore, incorporation of such elements in viral promoters could enhance the viral activity when oxygen supply is low.

Tcf4 is activated by β-catenin upon Wnt signalling, a pathway deregulated in virtually all colon cancers (see below). The laboratory of Dr. Iggo was the first to describe replicating adenoviruses that target colon cancer cells, where targeting is achieved by insertion of binding sites for Tcf transcription factors in early viral promoters.[34] The first viruses contained Tcf binding sites in their E2 and E1B promoters. E2 was chosen because of the absolute requirement for E2 gene products in virus replication. E1B was mutated in order to reduce inflammation *in vivo* and had no effect on virus growth *in vitro*.

I further developed these replicating adenoviruses by placing E1A under the control of a Tcf promoter, either alone or in combination with other mutant promoters. Multiply mutated viruses show increased selectivity towards the Wnt signalling pathway but are attenuated in some

colon cancer cell lines.[82] Other groups have targeted colorectal cancer with Wnt-dependant adenoviruses, which express the apoptotic gene *fadd* or prodrug converting enzymes such as nitroreductase and HSV thymidine kinase from a Tcf promoter.[47,146,155] As these vectors are non-replicating, they have less potential for further clinical development than replicating vectors (see chapter 1.2.1).

1.2.3. Cellular binding

The third kind of modification for converting the virus into an effective therapeutic agent was performed on the capsid proteins of adenoviral vectors. Expression of the native cellular receptor CAR is often reduced in tumour cells. CAR is an adhesion molecule and therefore it is conceivable that its down-regulation helps the dissemination of invasive tumours. Besides, CAR is present in many organs, including lung and liver. CAR binding, as achieved by the wild-type capsid, favours the targeting of normal cells.

Ablation of CAR and integrin binding decreases viral gene expression in liver but does not decrease hepatic viral uptake.[250] This is of specific interest, as approximately 90% of the injected viruses are found in the liver after intravenous injection and hepatotoxicity is one of the major hurdles towards systemic delivery of any therapeutic virus.[12] This suggests that additional mechanisms are present *in vivo*. Other factors have been proposed, like binding of the shaft domain of the fibre to heparan sulfate proteoglycans[221] or binding *via* Factor IX.[218] Kupffer cells are liver-resident macrophages that play an important part in adenovirus-induced inflammation, vector persistence, and transgene gene expression.[145,154,216] Depletion of these cells however does not decrease the number of viral genomes in the liver. This suggests that Kupffer cells are not a major factor underlying liver tropism.[160]

Various strategies have been followed for viral retargeting, and the most popular approaches were the deletion of the CAR-binding site and the insertion of specific peptide sequences into the knob domain of the fibre. This was done either at the C-terminus of the fibre[169,252] or in the HI loop, an

exposed and flexible region of the fibre knob.[61] The insertion of an RGD sequence in the HI loop facilitates binding and entry *via* integrins that are abundantly expressed on tumour cells.[61] An alternative approach to extend the tropism of adenoviruses is pseudotyping, where the fibre knob is replaced with one from another, non-CAR binding serotype.[110] In principle, any sequence able to trimerise in a coiled-coil fashion can be fused to the fibre tail to generate a chimeric fibre. Such strategies have been used to insert the reovirus fibre[214,241] or the phage T4 fibritin,[141] which show CAR-independent infection and retargeting to the novel receptor. The length of the shaft has also been reported to play a role in viral infection. Both shorter[6] and longer[217] shafts reduce liver transduction, most likely due to the length and the flexibility of the fibre which must allow the penton base to bind integrins while the knob domain is bound to CAR.[258] Another explanation is that long-shafted viruses cannot diffuse through the fenestrated endothelium of the liver. Other viral coat proteins have been mutagenised and ligands have been successfully added to penton base,[67] hexon,[247] and pIX proteins.[62,245]

Retargeting can also be achieved via conjugate-based strategies, where a molecule is used to bridge the viral capsid to a cellular surface protein. Bispecific antibodies that target both the fibre knob domain and a cellular receptor have been shown to redirect the tropism of the virus.[101,171] A similar strategy consists of using the fibre-binding domain of CAR[133] fused to a peptide[180] or a natural ligand like EGF.[60] In addition, binding of ligands to viruses can be achieved by biotinylation and bridging of the two reagents. Taken together, the disadvantage of these strategies is the need to manufacture and combine multiple reagents.

Another scheme to link targeting ligands to adenoviruses involves coating of the virion with polymers like polyethylene glycol (PEG)[56] or poly-[N-(2-hydroxypropyl)methacrylamide] (pHPMA).[74] These polymers can be coupled to peptides for re-targeting. Addition of a ligand through PEGylation was shown to expand viral tropism, but the effect on the ablation of the native tropism differs from study to study. In general, the native tropism was not ablated,[56,204] although a recent study has shown detargeting with PEGylated viruses.[148] In contrast, pHPMA coating *per se*

was shown both *in vitro* and *in vivo* to efficiently ablate viral uptake into cells. Addition of ligands onto the polymer coat enabled virus re-targeting, and pHPMA coating protected the virus from neutralising antibodies. With a manufacturing process that is simpler than the one involving bi-specific polypeptides, polymer coating represents a promising method for fast viral re-targeting.

1.2.4. Suicide gene therapy

Clinical trials have shown that oncolytic agents are not potent enough when used in monotherapies. In general, infection of tumour cells is inefficient or insufficient to lead to complete tumour regression. It is possible to express an unconditional toxin like TNFα[166] or diphteria toxin A[157] from a virus in order to increase its toxicity, but this raises biosafety concerns in a replicating human virus. A more prudent strategy is to express a prodrug-activating enzyme, where a prodrug is converted into its toxic form only in cells that express the transgene. The advantage of this approach is that the toxic effect is limited to the period of exposure to the prodrug, which itself is harmless. Initially, such genes were placed under the control of a ubiquitous early promoter like CMV or RSV,[48] but expressing these genes only in tumour cells decreased systemic toxicity and increased the therapeutic index of the viruses.[266]

The enzymes most commonly used for prodrug activation are HSV thymidine kinase (tk) and *E.coli* or *S.cerevisiae* cytosine deaminase (CD), although many other prodrug activating enzymes are being studied.[97,136] Thymidine kinase converts gancyclovir (GCV) to GCV monophosphate. Subsequent reactions catalysed by cellular enzymes lead to a number of toxic metabolites. Amongst them, GCV-triphosphate competes with deoxyguanosine triphosphate for incorporation into elongating DNA during cell division, causing inhibition of the DNA polymerase and single strand breaks.[98] Cytosine deaminases convert the prodrug 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU). 5-FU is a widely used cytotoxic drug which is metabolised to fluorinated ribo- and deoxyribonucleotides which have multiple effects, including inhibition of thymidylate synthase, altered DNA

stability, induction of single- and double-strand breaks, and decreased RNA stability.

In devising a strategy for expression of a suicide gene, one can choose between use of an early and a late transcription unit. Early expression risks killing the virus if the drug interferes with viral DNA replication.[79,203] Nevertheless, viruses expressing a CD-tk fusion gene under a heterologous promoter in the E1B55K region express high level of CD-tk and combination virus, prodrug and radiation therapy has given promising results in preclinical studies and in a phase I clinical study in prostate cancer.[78] Late expression of suicide genes is more attractive because replication can increase the number of transcription templates to many thousands of copies. Provided viral replication is restricted to tumour cells, genes expressed from late promoters should also be restricted to tumour cells. Therefore, there is no *a priori* reason to use a tumour specific promoter. Expression of a late gene is possible by making a fusion protein, by splicing a new exon into an existing transcript[112] or by reinitiation of translation from an internal ribosome entry site (IRES).[83,213]

1.3.   Wnt pathway

The Wnt signalling pathway is evolutionary conserved from nematodes to vertebrates (see Figure 1.4 for an overview of the pathway). This pathway is active during embryogenesis, where it has been shown to regulate many patterning events, like the formation of the dorso-ventral axis in the developing vertebrate embryo.
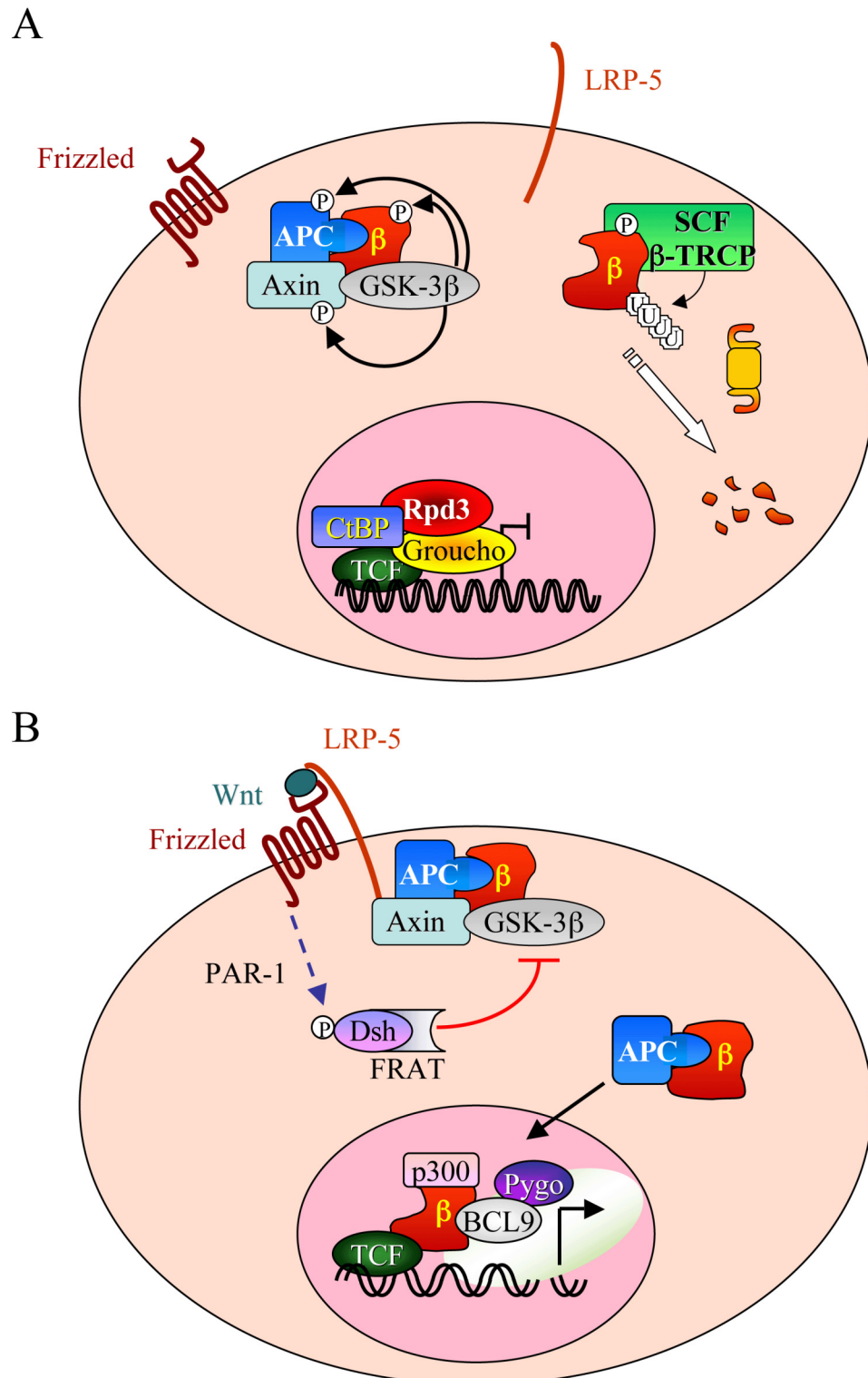
Figure 1.4: Overview of the Wnt signalling pathway. (A) In absence of a Wnt signal, β-catenin is phosphorylated by GSK-3β, ubiquitinated by the SCF$^{\beta\text{-TrCP}}$ complex, and targeted for degradation. Tcf/Lef transcription factors actively repress transcription by recruiting corepressors like histone deacetylases. (B) In presence of a Wnt signal, the destruction complex is destabilised and β-catenin can act as a cofactor for Tcf/Lef transcription factors.

In the adult body, the Wnt pathway is particularly linked to stem cell compartments, such as bone marrow, hair follicles and crypts of the intestine. It is not clear whether Wnt is important for the maintenance of the stem cell compartment or for the amplification of the transiently amplifying cells. In the intestinal crypts, activation of the Wnt pathway leads to expression of c-myc, which blocks the transcription of the cell-cycle kinase inhibitor (CKI) p21. This enables the cells to progress through the cell cycle.[244]

Wnt ligands can activate several pathways, namely: the canonical Wnt pathway, the planar cell polarity pathway, and the Wnt/Ca^{2+} pathway. These pathways branch early in the signalling cascade and are modulated by various proteins and protein-protein interactions.[129] In my thesis, I will focus on the canonical Wnt pathway, which is mutated in many cancers. In this pathway, binding of Wnt to its receptors induces a cascade of events that leads to the stabilisation of β-catenin and its translocation into the nucleus (Figure 1.4b). There, it functions as a cofactor for transcription factors of the T-cell factor/lymphoid enhancing factor (Tcf/Lef) family to turn on expression of target genes such as c-myc, cyclin D1, and axin2. Besides its role in the Wnt signalling pathway, β-catenin is involved in the formation of adherent junctions and links E-cadherin to α-catenin, which in turn binds to the actin cytoskeleton. In conclusion, β-catenin is distributed into three main cellular pools: membrane-bound, cytoplasmic and nuclear. The first pool participates in adherent junctions while the two others are mostly devoted to regulation of the Wnt signalling pathway.

1.3.1. Molecular mechanisms of the Wnt signalling pathway

In absence of Wnt signal, β-catenin is part of a multiprotein cytoplasmic complex, called the "destruction complex", that contains APC, axin, CKI and GSK3β (see Figure 1.4 for an overview of the pathway, Figure 1.5 for a schematic description of the main proteins,[182] and Giles, 2003 for a recent review[90]). APC is a large protein that interacts with many proteins including β-catenin, axin, EB-1 and DLG, and is capable of

homodimerisation. It has multiple roles in cell migration, adhesion, spindle orientation, cell cycle regulation and chromosome stability.[189] APC exports β-catenin to the cytoplasm and targets it to the destruction complex.[122,207] The critical role of APC in tumorigenesis is linked to its capacity to down-regulate cellular levels of β-catenin (see chapter 1.3.3.). In the adult body, APC is mainly expressed in regions where cell replication has ceased and terminal differentiation is established.[170]
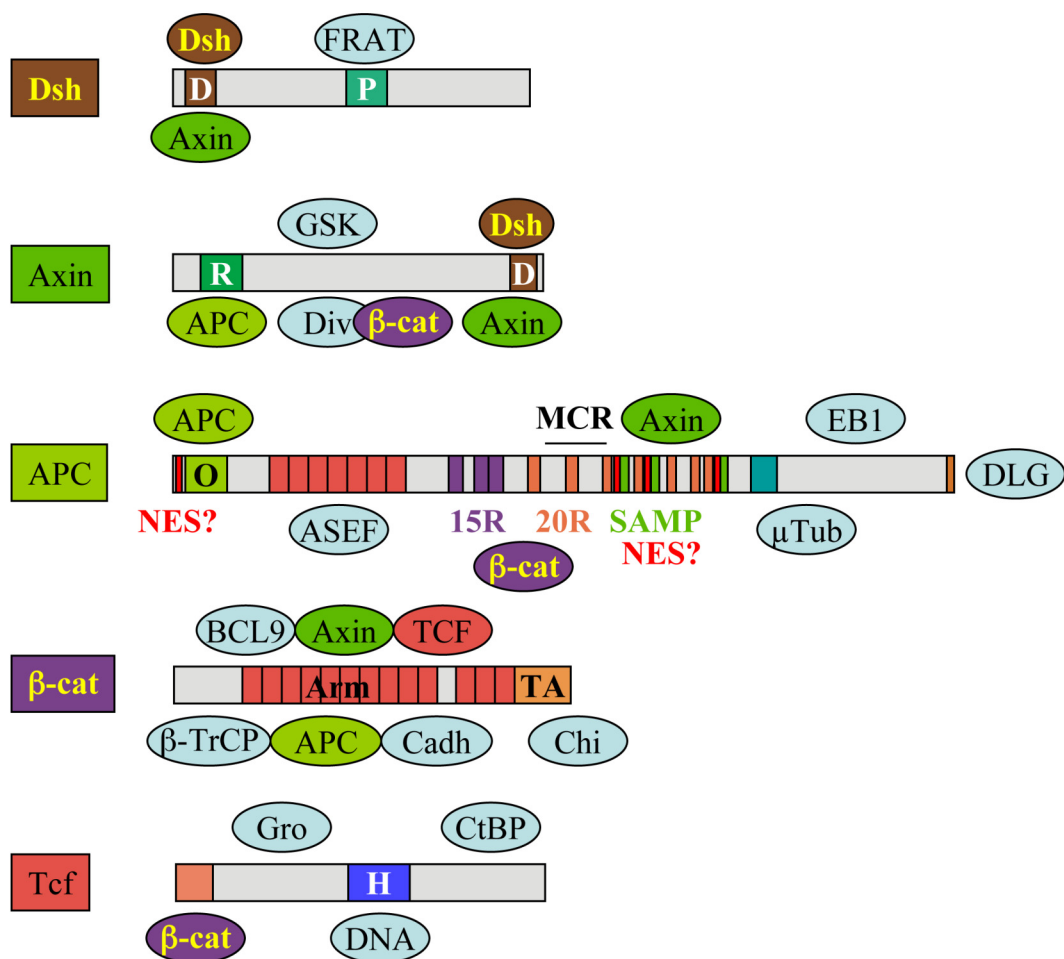

Figure 1.5: Structure of the proteins of the Wnt signalling pathway. Arm=armadillo repeats, TA= Transactivation domain. O=oligomerisation domain. P=PDZ domain. D=DIX domain. R=RGS domain. H=HMG domain. 15R, 20R=β-catenin interaction domains. SAMP=axin-binding sites. NES=nuclear export signal. MCR = mutation cluster region. Div = diversin. Gro = groucho. Cadh = Cadherin. Chi = Chibby. [182]

Axin acts as a scaffold protein that brings together GSK3β, APC, β-catenin, and diversin.[128] This complex stimulates a two-step phosphorylation of β-catenin. First, Casein Kinase I (CKI) binds diversin and phosphorylates β-catenin at Ser-45. Then, once primed by CKI, β-catenin is phosphorylated on the remaining N-terminal sites by GSK3β,[7,159] which can also phosphorylate axin to increase the stability of the destruction complex.[259]

β-catenin is a poor substrate for "free" GSK3β and can only be efficiently phosphorylated within the destruction complex. Polyphosphorylated β-catenin is then recognised by the F-box β-TrCP and degraded *via* the ubiquitin/proteasome pathway.[158] In the nucleus, Tcf transcription factors are bound to their target DNA sequence and to transcriptional repressors of the groucho family, which in turn recruit histone deacetylases to actively repress promoters by chromatin condensation.[46,205]

Canonical Wnt signalling is initiated upon binding of a Wnt ligand to a member of the Frizzled (Fz) family of receptors and to the co-receptor LRP–5/6.[20,192] Upon Wnt binding, the intracellular domain of LRP-5 interacts with axin and destabilises the scaffold protein[165]. In parallel, Wnt binding to Fz leads to phosphorylation of Dishevelled (Dsh),[260] which is followed by Frat-1-mediated dissociation of GSK3β from axin (Figure 1.4b). Once the destruction complex is destabilised, phosphorylation of β-catenin is stopped and the protein accumulates and translocates to the nucleus.

β-catenin contains 12 armadillo repeats that mediate its interaction with proteins such as cadherins, APC, axin, and Tcf. These repeats are sufficient for nuclear import.[71] Nuclear sequestration has also been shown to be mediated by APC *via* the APC/β-catenin complex.[178] Once in the nucleus, β-catenin binds to Lef/Tcf transcription factors and to additional proteins implicated in histone acetylation and remodelling such as CBP/p300[119,233] and Brg-1.[11] Furthermore, β-catenin activation is regulated by BCL9 and Pygopus through an unknown mechanism.[140] Active β-catenin-Tcf complexes drive transcription of target genes such as c-Myc,[116] CyclinD,[234] MMP-7,[28] PPARδ,[115] and axin2/conductin.[161]

1.3.2. Colorectal cancer and Wnt

Colorectal cancer (CRC) is a leading cause of mortality with about 300'000 new cases and 200'000 deaths in Europe and in the USA each year. Most colorectal cancers arise within pre-existing adenomatous polyps or adenomas, which are common lesions. Studies have shown a prevalence of about 35% in Europe and in the USA, with lower rates in Asia and Africa (10-15%). About 5% of adenomatous polyps are estimated to become malignant, a process that takes 5 to 10 years.[181]

The risk of developing colorectal cancer is determined by genetic predisposition and environmental factors such as diet. Hereditary forms of CRC are familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC). FAP patients have a mutant APC gene, whereas HNPCC patients have mutations in genes involved in DNA mismatch repair.

Each cell must undergo multiple mutations for malignant transformation to occur. Vogelstein and colleagues have proposed a model where multistep genetic events favour the progression of sporadic CRC. These events involve oncogenes like K-Ras and myc and tumour suppressor genes like APC and p53.[73] Remarkably, virtually all CRCs have activating mutations of the canonical Wnt signalling pathway, leading to stabilisation and nuclear accumulation of β-catenin. The vast majority of sporadic colon cancers (85%) have truncated APC proteins and both alleles of APC need to be mutated for loss of growth-suppressing activity. When the first mutation occurs close to codon 1300 (before the axin-binding sites), the second allele usually undergoes loss-of-heterozygosity. On the other hand, when the mutations are outside this region, the second allele is often mutated in that particular region. This suggests that the best selective advantage for cancer cells is achieved by retention of a single truncated APC allele that retains the ability to bind to β-catenin but fails to bind to axin. In theory, such a protein is still able to facilitate the nuclear import of β-catenin, but is unable to export and degrade it (see chapter 2.2).

The remaining 10% of the CRC tumours have mutations that affect the phosphorylation sites of β-catenin, thus making it untargetable for

degradation. APC and β-catenin mutations are mutually exclusive, consistent with their redundancy in the activation of the Wnt pathway. However, β-catenin mutations are under-represented in invasive cancers and seem less potent than APC mutations to progress from small adenomas to invasive carcinomas. This suggests that both mutations are not functionally identical and that APC is the preferred target for mutations in the Wnt pathway.[90]

Mouse models of heterozygous mutations for truncated alleles of APC mimic more or less the phenotype encountered in FAP patients and develop numerous adenomas throughout the gastro-intestinal tract.[228] Genotyping of these early lesions showed that all had lost their wild-type APC allele, indicating that the mutation in APC is an early event in tumour initiation.[186] Mice harbouring a truncated APC that can still degrade β-catenin remain tumour-free.[222] Stem cells homozygous for this mutation however exhibit chromosomal instability, underlining the role of APC in chromosomal segregation.[75] Mutation in APC is of particular importance to CRC as it results to lack of β-catenin degradation and improper segregation of chromosomes. This links the activation of the Wnt pathway to chromosomal instability (CIN), a phenomenon observed in most of the APC mutant cells.

1.4. Pre-existing Tcf-viruses

As the Wnt pathway is predominantly switched off in the adult body but constitutively activated in most colorectal cancer, Wnt-targeting viruses should specifically target colon cancer cells. In order to target the Wnt pathway, Tcf-binding sites were inserted in endogenous viral promoters to activate the transcription of essential viral genes, a project initiated by M. Brunori in the lab of Dr. R. Iggo. The first viruses included Tcf binding sites in the E2 promoter as well as silent mutations in the nearby E3 promoter. The E2 promoter was chosen because mutations elsewhere in the virus or cell cannot bypass the absolute requirement for E2 gene products in virus replication, and E3 was mutated to avoid cross-talk between the two promoters. Tcf sites were also inserted in the E1B promoter. This reduced

inflammation *in vivo* but had no effect *in vitro*.[34] Although these viruses are approximately 100-fold selective for cells with an activated Wnt pathway, several colon cancer cell lines were found to be only weakly permissive for the Tcf-viruses, despite harbouring activating mutations in the Wnt pathway. These cell lines are called semi-permissive. During the course of my Ph.D. studies, I focused on the improvement of Tcf-targeting vectors and on the difference between permissive and semi-permissive cell lines.

2.   Results and discussion

2.1.   Summary

To increase the selectivity of the Tcf-viruses, I placed the E1A gene under the control of a tightly regulated Tcf promoter. To achieve this, I deleted the region ranging from the second half of the ITR to the TATA box and replaced it by four consensus Tcf binding sites. To ensure proper encapsidation of the virus, the packaging signal was inserted at the right end of the genome. In addition, in order to maintain the symmetry needed for proper viral DNA replication, three Tcf binding sites were inserted in the right ITR. I showed by reporter assays that the E1A promoter is regulated by the Wnt pathway and that it is more active than the endogenous promoter. In contrast, the E4 promoter was shown to be less active than the wild-type promoter. Cotransfection experiments showed that the E1A 12S protein has an inhibitory effect on the Tcf-promoters. I showed by cotransfection of various E1A deletion mutations that E1A inhibits Tcf-dependent transcription partly through sequestration of p300, which at the time was shown to be a co-factor of β-catenin.[119,233]

To characterise the effect of the mutations in the E1A and E4 promoters in the context of the viral genome, I constructed viruses containing the E1A/E4 changes in either wild type or Tcf-viruses. My results showed that the activity of the E1A promoter depends on an activated Wnt pathway, and that selective expression of E1A leads to selective production of progeny virus and viral spread. The activity and selectivity of the Tcf-E1A/E4 virus was comparable to that of the Tcf-E2 viruses. Therefore, a Tcf-driven E1A promoter confers selective viral replication in cells with an active Wnt pathway. Viruses containing Tcf binding sites in the E1A, E1B, E2, and E4 promoters showed enhanced selectivity (100'000-fold) for cells with an active Wnt pathway. Deletion of the p300-binding domain of E1A in the context of a virus did not lead to increased viral activity but resulted in the attenuation of the virus.[82]

It had been shown that the cellular permissivity to the Tcf-viruses correlates with their Wnt activity, but cotransfection of members of the Tcf/β-catenin complex failed to increase the activity of a Tcf-promoter in various colon cancer cell lines. This suggests that the reduced Wnt activity might be the consequence of something either located downstream of β-catenin, or regulating β-catenin stability or localisation. Cotransfection or stable expression of the downstream activators BCL9 and Pygopus showed that these factors could not be held responsible for the difference between semi-permissive and permissive cell lines.

Analysis of mutations in β-catenin or APC, as well as β-catenin localisation studies, indicated that the presence of an APC protein capable of exporting β-catenin to the cytoplasm correlates with semi-permissivity. The Tcf-E2 promoter was shown to be the most sensitive to cell type and Tcf-viruses failed to induce host cell protein shut-off in a semi-permissive cell line. To improve the activity of the Tcf-E2 promoter, I did targeted mutagenesis but failed to see any improvement in reporter assays. Because reporter assays might not reflect the behaviour of the promoter in the context of the viral genome, I constructed a virus that will permit screening of multiple E2 promoters.

Another way to increase the activity of the Tcf-E2 promoter is to bind a transactivator to β-catenin. The virus uses the E1A protein to bridge transcription factors like ATF to components of the mediator, a multiprotein complex involved in transcription activation. The HD2 domain of BCL9 was shown to bind to β-catenin without disrupting the β-catenin/Tcf interaction. Therefore, an E1A-HD2 fusion protein was constructed to bridge E1A to β-catenin during adenoviral infection. Because reporter assays had shown that E1A-HD2 can increase the activity of a Tcf promoter in a β-catenin dependent manner, I constructed an E1A-HD2 virus that might have increased activity in cells with an activated Wnt pathway.

Finally, to increase the toxicity of the existing Tcf viruses, I expressed yeast Cytosine Deaminse (yCD) with late kinetics in a Tcf-E1A/E4 backbone. yCD is expressed in these viruses either through re-initiation of translation after the fibre or by alternative splicing. I showed that yCD is

expressed late during infection, and that its expression is dependent on viral DNA replication, following the expression kinetics of endogenous late genes. Treatment of infected cells with the prodrug 5-FC had little effect on viral replication but increased the cytotoxity of the Tcf-viruses. The yCD viruses showed a slightly increased activity in non-permissive cell lines, due to expression of early viral proteins through an unknown mechanism, but retained their selectivity for cells with an activated Wnt pathway.[83]

2.2. Adenoviruses used in this study

In this thesis, the viruses will be described with a codename summarising their structure. For example, a Tcf-E1<u>A</u>/E<u>4</u> virus will be described as the "A4" virus. All the viruses used in this study are shown schematically in the figure below, and the mutations are listed in the legend.
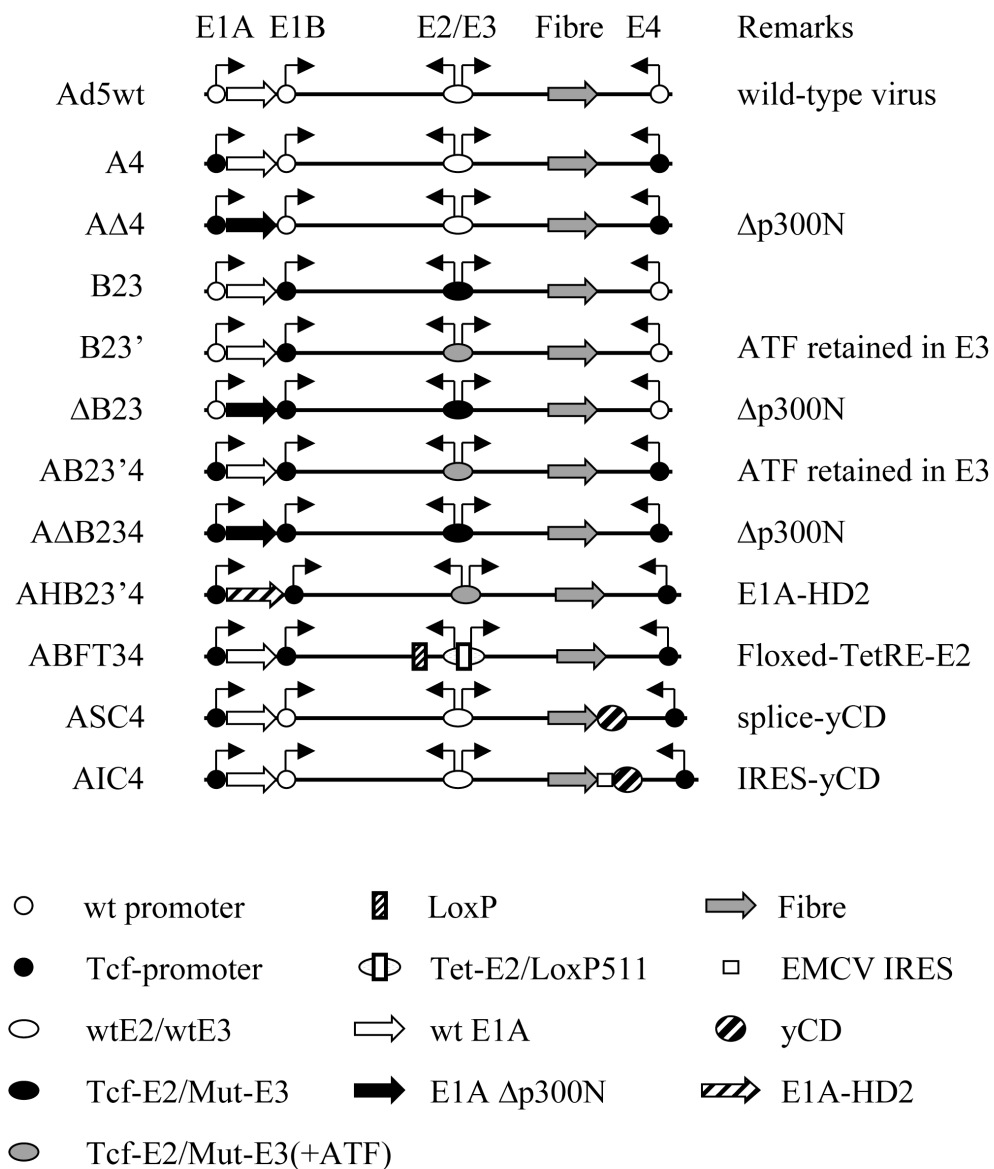
	E1A E1B	E2/E3	Fibre E4	Remarks
Ad5wt				wild-type virus
A4				
AΔ4				Δp300N
B23				
B23'				ATF retained in E3
ΔB23				Δp300N
AB23'4				ATF retained in E3
AΔB234				Δp300N
AHB23'4				E1A-HD2
ABFT34				Floxed-TetRE-E2
ASC4				splice-yCD
AIC4				IRES-yCD

○ wt promoter   ▧ LoxP   ⇨ Fibre

● Tcf-promoter   Tet-E2/LoxP511   □ EMCV IRES

◯ wtE2/wtE3   ⇨ wt E1A   ⊘ yCD

⬤ Tcf-E2/Mut-E3   ➡ E1A Δp300N   E1A-HD2

⬭ Tcf-E2/Mut-E3(+ATF)

Figure 2.1 Schematic diagram showing mutant regions in the viruses used in this study. To facilitate interpretation of the figures, the viruses are given a codename summarising their structure: A, B, 2, 4 = Tcf sites in the E1A, E1B, E2, and E4 promoters, respectively. 3 = silent mutations in the NF1, NFκB, AP1, and ATF sites in the E3 promoter. 3' = as 3, but without the ATF site mutation. Δ = deletion of amino acids 2-11 in E1A that abolishes p300 binding. H = E1A-HD2. FT = Floxed- and Tet-regulated E2 promoter. C = yCD coding sequence. I = EMCV IRES. S = Ad41 long fibre splice acceptor sequence.

2.3. Adenoviruses with Tcf-binding sites in multiple early promoters

2.3.1. Introduction

The group of Dr. Iggo was the first to describe adenoviruses that replicate in response to activation of the Wnt signalling pathway.[34] The rationale for the development of these viruses was that Wnt signalling is pathologically activated in virtually all colon tumours,[193] leading to activation of promoters containing Tcf binding sites.[138] Originally, Tcf binding sites were inserted in the adenovirus E2 promoter because mutations elsewhere in the virus or cell cannot bypass the absolute requirement for E2 gene products in viral replication. In order to achieve tight regulation of E2 transcription, the adjacent E3 enhancer was mutated. In addition, Tcf sites were inserted in the E1B promoter, but this particular change did not affect viral replication *in vitro*. The Tcf-E2 viruses showed a 50- to 100-fold decrease in replication in non-permissive cell lines whereas their activity was comparable to wild type Ad5 in many but not all colon cancer cell lines.[34] The remaining colon cell lines were semi-permissive for the Tcf-viruses.

In this chapter, I will describe the two approaches I initially followed to render the existing Tcf viruses active in a broader range of colon cell lines. I first inserted Tcf-binding sites in the E1A and E4 promoters and showed that the activity of the Tcf-E1A promoter was Wnt-dependent, whereas the activity of the Tcf-E4 promoter was reduced in a non-specific manner. Second, I mutagenised the E1A protein to determine which of its domains represses β-catenin-dependent transcription, and mapped this domain to the p300-binding domain of E1A. Mutation of this domain partially relieved E1A-mediated repression of Tcf-dependent transcription in reporter assays. Nevertheless, in the context of the virus, this Δp300 mutation did not lead to increased transcription from the Tcf-E2 promoter and actually reduced the viral activity. Similar attenuation by mutation of the amino-terminus of E1A had been reported by the Onyx group.[120] Mutation of all early promoters resulted in highly selective, yet attenuated

viruses. The new virus containing only the Tcf changes in the E1A and E4 promoters (A4) was selective for cells with active Wnt signalling and active in most of the colon cancer cells studied.

2.3.2. Results

Mutation of the E1A and E4 promoters

The wild type E1A enhancer contains two types of regulatory element, termed I and II,[117] which overlap the packaging signal. In addition to these elements, there are transcription factor binding sites in the inverted terminal repeat (ITR) and close to the E1A TATA box (Figure 2.2a, E1Awt). To produce a tightly regulated E1A promoter responding only to Wnt signals, the E1A enhancer, the packaging signal, and half of the ITR were deleted and replaced with four Tcf binding sites. The changes in the ITR do not affect the minimal replication origin.[41] The resulting E1A promoter contains four Tcf sites and a TATA box (Figure 2.2a, E1Amut). To maintain the symmetry of the terminal repeats and preserve the ability of the two ITRs to anneal during viral DNA replication, three Tcf sites were inserted in the right ITR. The packaging signal was also inserted at the right end of the genome to permit proper encapsidation of viral DNA (Figure 2.2b, E4mut).
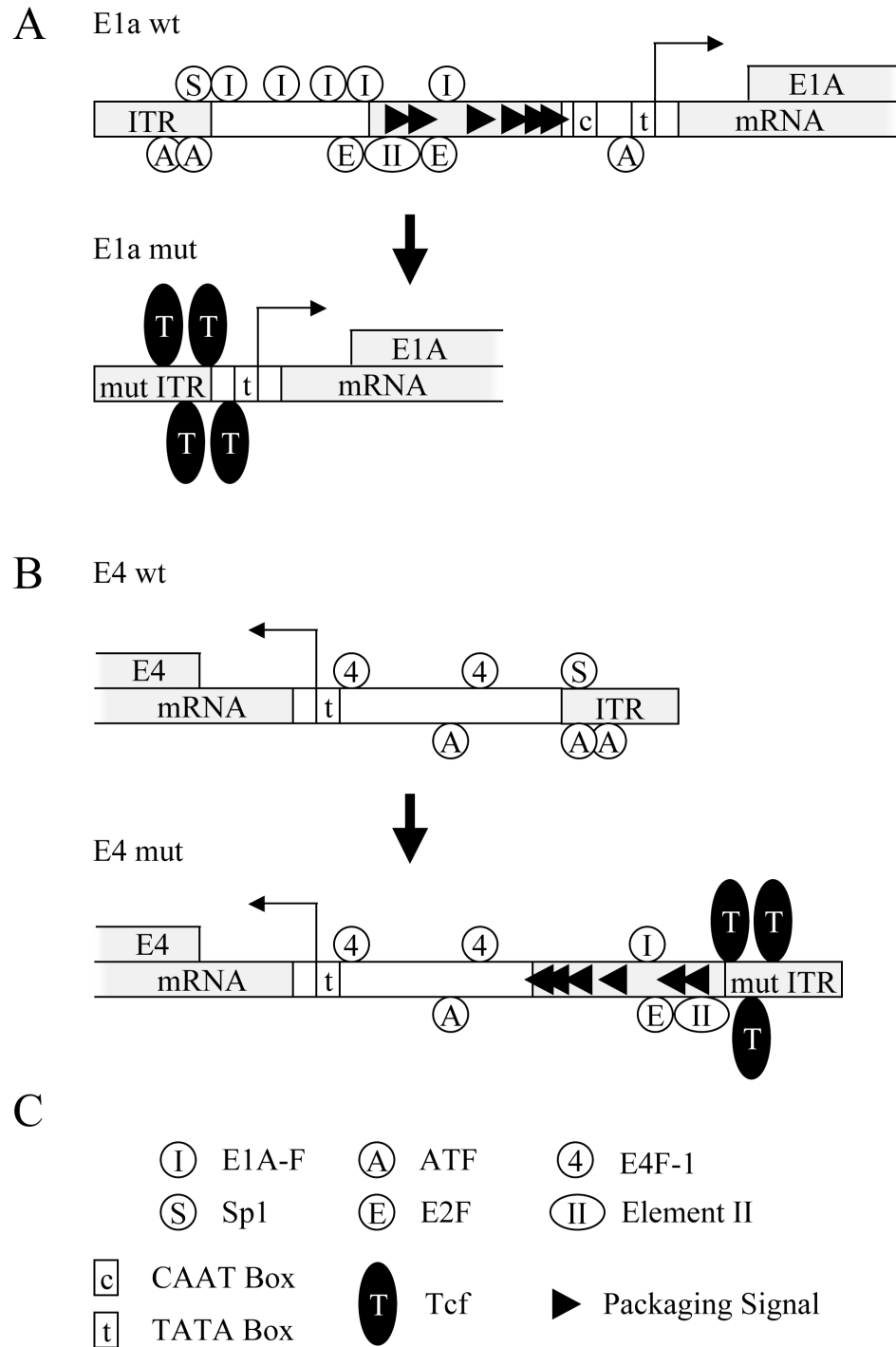
Figure 2.2: Schematic diagram showing the mutagenesis of (A) the E1A promoter, and (B) the E4 promoter. Both regions are shown from the ITRs to the beginning of the first open reading frame. (C) Legend. The dark triangles represent the A motifs in the packaging signal.

The promoters were tested in reporter assays (Figure 2.3) in H1299 and SW480. H1299 is a human lung cancer cell line that does not contain activating mutations in the Wnt pathway. SW480 is a colon cancer line with

a truncated APC. In H1299, the basal activity of the mutant promoter is lower than that of the wild-type promoter. Upon cotransfection of ΔN-β-catenin, the E1Awt promoter was only slightly activated in H1299 (Figure 2.3a; lanes labelled "β"), whereas the Tcf-E1A promoter was activated about 10-fold to reach a final level that was 2-fold that of the wild-type promoter. This suggests that the Tcf-E1A promoter should confer selectivity for cells with an active Wnt pathway when incorporated into adenoviruses.
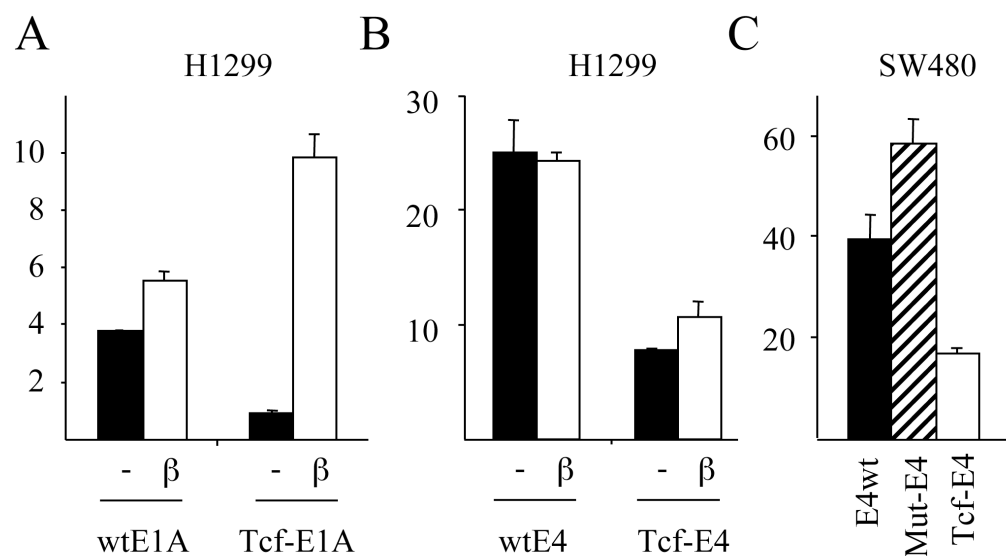
Figure 2.3: Activity of the E1A and E4 promoters measured by luciferase reporter assay. (A) Activity of the wild type or Tcf- E1A promoter in H1299 either in absence (-) or in presence (β) of stabilised β-catenin. Activity of the various E4 promoters in H1299 (B) or SW480 (C). The Mut-E4 promoter has three Tcf binding sites but no encapsidation signal. Values are expressed in arbitrary units on a linear scale.

The mutant E4 promoter (Tcf-E4) was not regulated by the Wnt pathway (Figure 2.3b; compare "–" and "β"). Surprisingly, its activity was lower than that of the wild-type E4 promoter, even in the presence of ΔN-β-catenin, or in the permissive cell line SW480 (Figure 2.3c). The Mut-E4 promoter contains the three Tcf binding sites in the ITR but does not have the encapsidation signal. This promoter was more active than the wild-type promoter in SW480 cells (Figure 2.3c, compare Mut-E4 with E4wt). This suggests that insertion of Tcf-binding sites in the E4 promoter confers some Wnt-inducibility, although this was not directly tested. Addition of the packaging signal in Tcf-E4 led to a 3-fold decrease in promoter activity

(Figure 2.3c, compare Tcf-E4 with Mut-E4). Such a loss in activity was unexpected as the packaging signal contains some enhancer sequences of the E1A promoter[117]. It is possible that the localisation of these enhancer sequences within the E4 promoter is not compatible with proper activation of transcription. Moreover, insertion of the packaging signal increased the distance between the Tcf binding sites and the TATA box, further preventing Tcf-dependent transactivation. Provided the Tcf-E4 promoter can sustain efficient viral activity, reduced activity may help to reduce hepatotoxicity, as E4 gene products have been reported to play an active role in liver damage.[51] In conclusion, this construct remains a valid candidate for the development of Tcf-viruses.

Tcf-E1A/E4 promoter viruses

Because of ITR symmetry and packaging signal requirements, both the E1A and E4 mutations must be present in the virus. Adenoviral genomic DNA was created by homologous recombination in yeast[86] using various adenoviral backbones such as wtAd5 or Tcf-E2 viruses.[34] The viral genomes were converted to virus in C7 cells[5] expressing a stable β-catenin mutant. Primary virus stocks were plaque purified and expanded on SW480 cells. The Tcf-E1A/E4 mutant viruses grew readily on SW480 cells, indicating that the ITR mutagenesis and exchange of the packaging signal are compatible with the production of viable virus. All the constructs are shown schematically in Figure 2.1 (page 40). Viruses are described with letters and numbers that correspond to the mutant promoters. For example, the Tcf-E1A/E4 virus is described as the "A4" virus (see legend of Figure 2.1 for further description of the abbreviations used in this study). To determine whether the Tcf-E1A promoter responds to activation of the Wnt pathway in the context of the virus, I infected cMM1 cells with A4 virus. cMM1 cells are a clone of H1299 lung cancer cells expressing ΔN-β-catenin from a tetracycline-regulated promoter.[164] Wnt signalling was activated by removal of tetracycline from the medium (Figure 2.4, lanes 5-8, ΔN-β-catenin). This had no effect on E1A expression by wild type Ad5, but induced expression of E1A by the A4 virus (Figure 2.4, lanes 3 & 7, E1A). Since DBP is

expressed from the normal E2 promoter in A4, the DBP level should rise following activation of Wnt signalling, because the normal E2 promoter is activated by E1A. The promoter was weakly active in the absence of E1A in H1299 cells, and showed a moderate and reproducible increase in activity following induction of ΔN-β-catenin expression (Figure 2.4, lanes 3 & 7, DBP). Thus, the mutant E1A promoter responds to activation of the Wnt pathway, and this feeds through to an effect on expression of viral replication proteins.
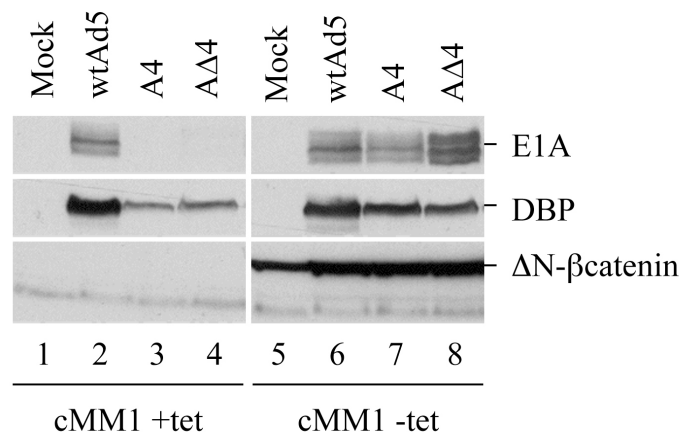

Figure 2.4: The Tcf-E1A promoter responds to activation of wnt signalling. Western blot of cMM1 cells probed for E1A and DBP 24 hours after infection with wild type Ad5 and Tcf-viruses. Tetracycline withdrawal leads to expression of ΔN-β-catenin in these cells ("-tet").

The effect of the Tcf-E1A/E4 promoter substitutions was then tested on a panel of colon cell lines with active Wnt signalling: SW480, ISREC-01 and HT29 have mutant APC; Hct116 has mutant β-catenin; and Co115 has microsatellite instability but the defect in Wnt signalling has not been defined.[55] Three control cell lines with inactive Wnt signalling were tested: H1299, HeLa and low passage normal human small airway epithelial cells (SAEC). E1A was detectable by western blotting 24 hours after A4 infection of all of the colon cell lines but not H1299, HeLa or SAEC (Figure 2.5, lane 3, E1A). Relative to wild type Ad5, the level of E1A expression was higher in SW480 and ISREC-01, the same in Co115 and lower in HT29 and Hct116 (Figure 2.5, compare lanes 2 & 3, E1A). The hierarchy of responsiveness of the Tcf-E1A promoter in the different cell lines was thus the same as with the Tcf-E2 viruses[34] but the level of expression relative to

the normal promoter was higher for E1A than E2. Since the E1B and E2 enhancers are wild type in A4, these transcription units should be inducible by E1A. The E4 promoter in A4 is potentially able to respond to both E1A and Tcf.
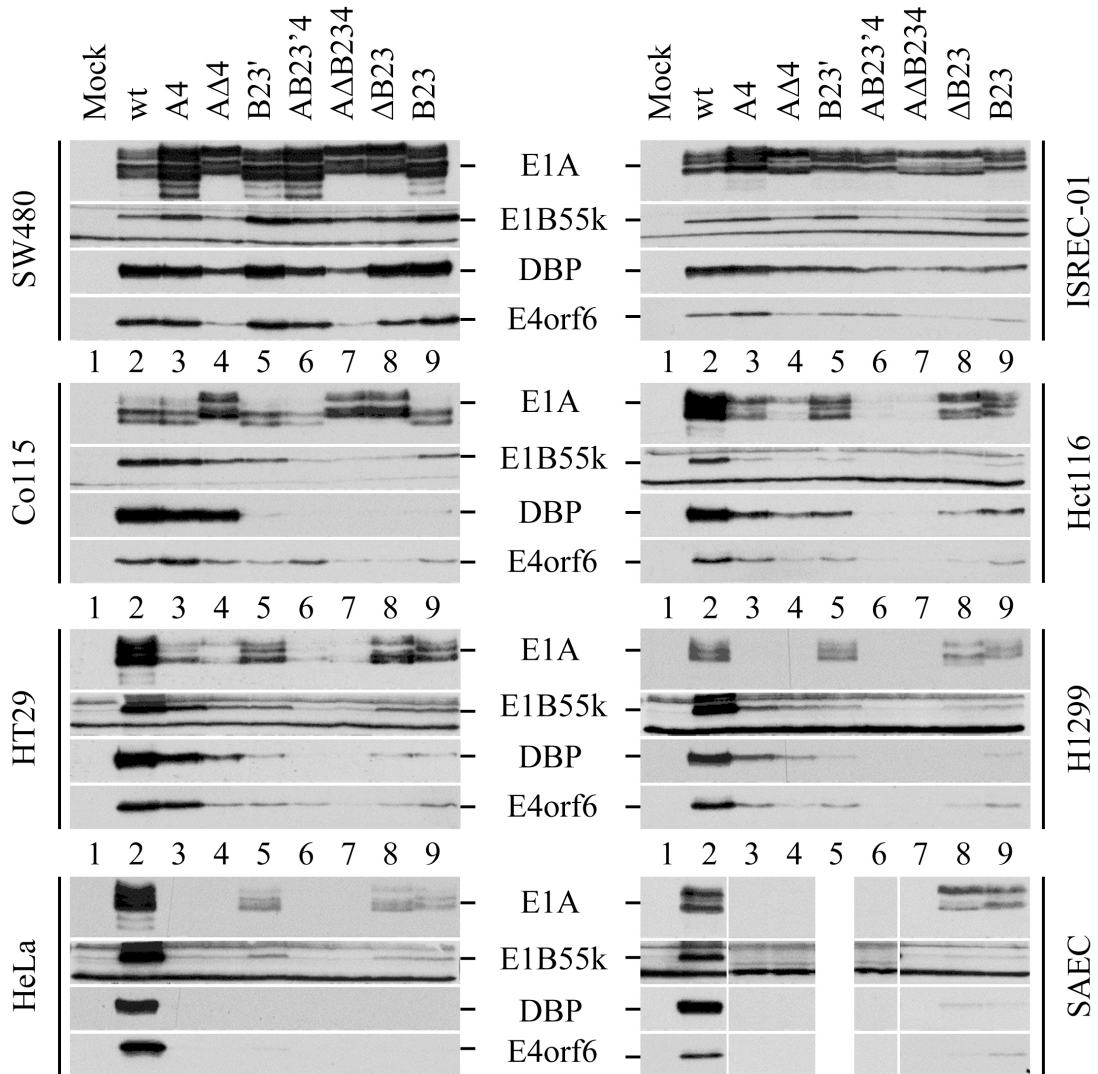

Figure 2.5: Western blot for E1A, E1B55k, DBP and E4orf6 24 hours after infection of different cell lines with wild-type Ad5 and Tcf viruses. SW480 and ISREC-01 are permissive colon cancer cell lines. Co115, Hct116 and HT29 are semi-permissive colon cancer cell lines. H1299, HeLa and SAEC are non-permissive cell lines in which the Wnt pathway is inactive. The SAEC blot is derived from two separate experiments giving similar wild-type Ad5 activity.

To test this, I tested the expression of the E1B55K, DBP and E4orf6 proteins. Consistent with the E1A results, all three proteins were expressed normally in SW480, ISREC-01 and Co115, and undetectable in HeLa and

SAEC (Figure 2.5, compare lanes 2 & 3). Despite the absence of E1A expression, all three proteins were expressed weakly in H1299 cells, suggesting that these cells contain an endogenous activity that can substitute for E1A. Compared to wild type infections, the levels of E1B55K, DBP and E4orf6 were slightly reduced in HT29 and more substantially reduced in Hct116 cells infected with A4 (Figure 2.5, compare lanes 2 & 3).

Viruses with Tcf sites in multiple early promoters

To test the effect of regulating E1A expression in the context of the previous generation of Tcf viruses, cells were infected with B23' and AB23'4 (Figure 2.5, compare lanes 5 & 6). E1A and E4orf6 expression were well preserved in SW480, ISREC-01 and Co115 infected with AB23'4, but DBP expression was maintained only in SW480 and ISREC-01, and even there it was slightly lower with AB23'4 than wild type Ad5 (Figure 2.5, compare lanes 2 and 6, DBP). In the remaining cell lines, DBP expression was undetectable with AB23'4. Reduction of E1A protein levels was seen in various cell lines even when E1A was expressed from its endogenous promoter (Figure 2.5, compare lanes 9 and 2, E1A), probably because lower DBP levels resulted in less viral DNA replication. Insertion of Tcf sites in the E1A, E1B, E2 and E4 promoters abolished the E1A-independent expression of E1B55K, DBP and E4orf6 seen in H1299 infected with A4 (Figure 2.5, compare lanes 3 and 6, H1299). In consequence, insertion of Tcf sites into multiple early promoters produces an extremely selective virus but one with reduced activity in some colon cell lines.

Inhibition of Tcf-dependent transcription by E1A

The defect in early gene expression from the Tcf viruses in the semi-permissive cell lines is not restricted to a single promoter. Instead, there appears to be a general defect in activation of viral Tcf promoters. This can be partly explained by generally weaker Tcf activity. The reason for this is unclear, but it does not reflect a lack of Wnt pathway activation *per se*,

since most of the semi-permissive cell lines contain mutations in either APC or β-catenin, and the Tcf-E2 transcriptional activity measured by luciferase assay is not increased by transfection of exogenous ΔN-β-catenin, hTcf4, combination of the two proteins, or a fusion protein between the transactivation domain of β-catenin and hTcf1 (β-cat-hTcf1; Figure 2.6). The differences between permissive and semi-permissive cell lines will be further examined in chapter 2.4.
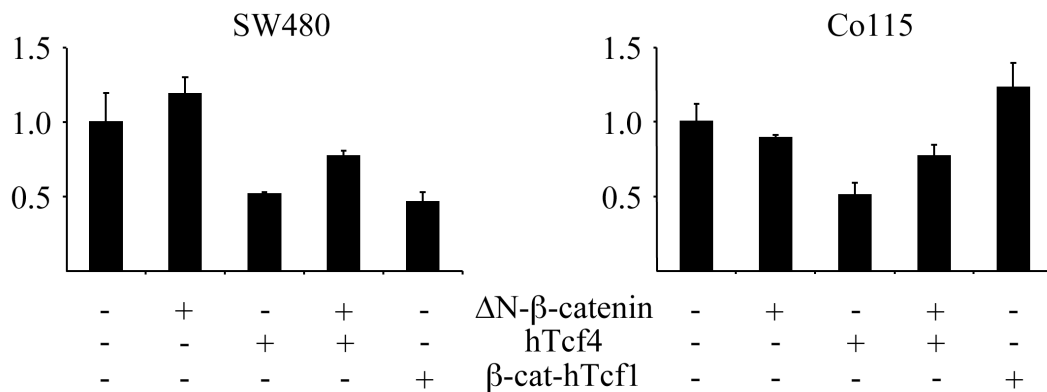

Figure 2.6: Members of the Tcf/β-catenin complex are not limiting in SW480 and Co115 colon cancer cell lines. Luciferase assays in SW480 and Co115 using a Tcf-E2 reporter. β-cat-hTcf1 is a fusion protein between the transactivating domain of β-catenin and hTcf1. The values are normalised to the activity of the Tcf-E2 promoter and expressed on a linear scale.

An alternative explanation for this semi-permissivity is that E1A could inhibit the viral Tcf promoters, for example, by sequestering p300, which is a coactivator of Tcf-dependent transcription.[119,233] To determine whether E1A inhibits the viral Tcf promoters, I performed transcription assays using the Tcf-E1A and Tcf-E2 promoters coupled to the luciferase gene. In SW480, the Tcf-E2 promoter was more active than the wild type E2 promoter in the absence of E1A (Figure 2.7b, lanes 1 & 6), and gave almost exactly wild type activity in the presence of E1A (Figure 2.7b, lanes 2 & 7). This convergence was due to increased wild type E2 promoter activity and decreased Tcf-E2 promoter activity in the presence of E1A. Mutation of the E3 promoter is required to produce a tightly regulated Tcf-E2 promoter, because the E3 promoter is adjacent to the E2 promoter.[34]
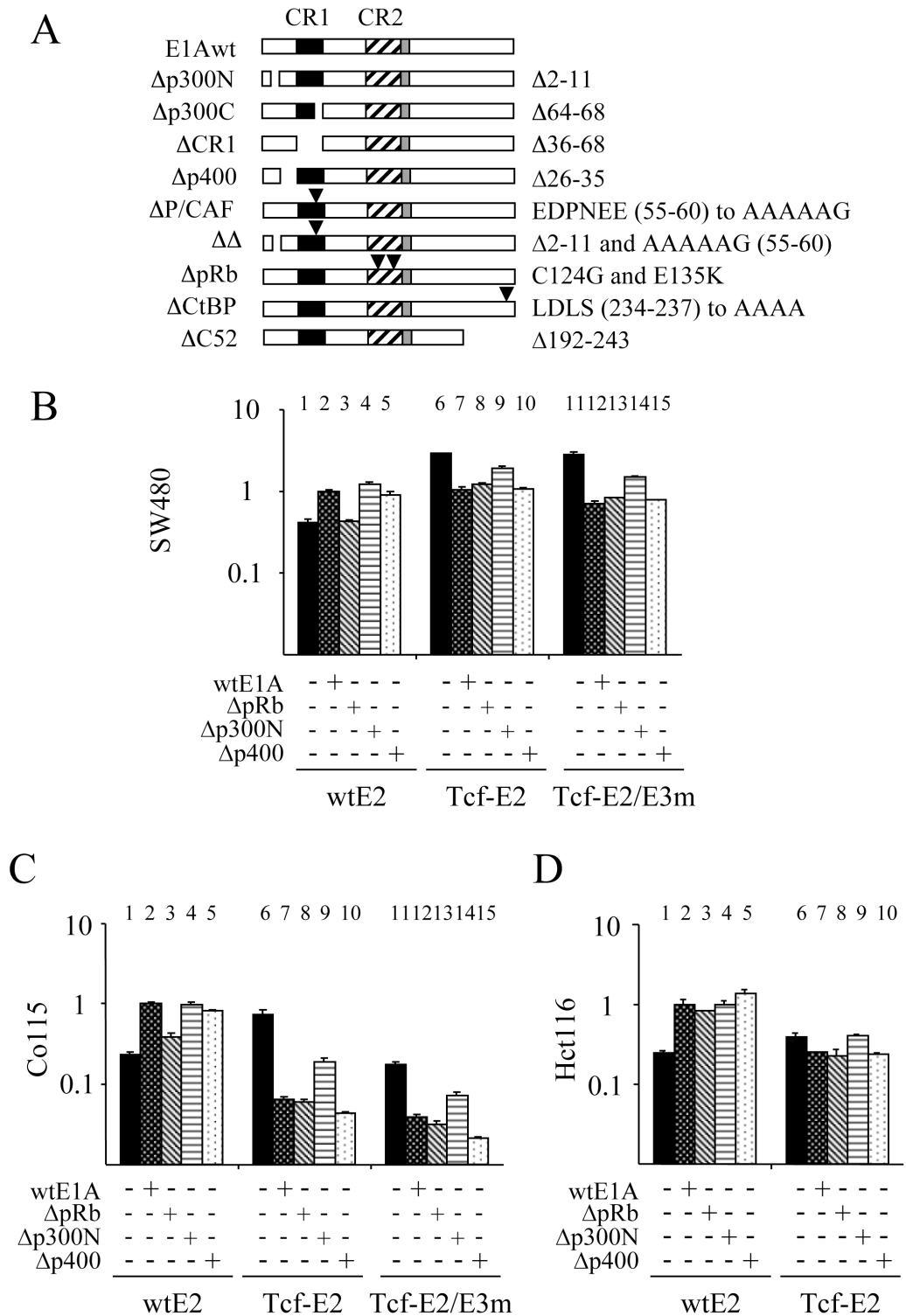
Figure 2.7: E1A inhibits Tcf-dependent transcription. (A) Schematic diagram of the E1A12S mutants. (B-D) Luciferase assays with a wild-type E2 reporter and Tcf-E2 reporters. The "Tcf-E2/E3m" reporter contains inactivating mutations in the E3 enhancer.[34] Cells were transfected with luciferase reporters and plasmids expressing the E1A mutants shown in A. (B) SW480, (C) Co115, (D) Hct116. Values are normalised to the value of the wild-type E2 promoter in presence of E1A.

E3 mutation reduced the activity of the E2 promoter slightly in SW480 cells transfected with E1A, but the activity was still close to that seen with the wild type promoter (Figure 2.7b, lanes 2 & 12). The high activity of the Tcf-E2 promoter in SW480 probably explains the permissivity of this cell line for the Tcf-viruses. In contrast, in the presence of E1A the level of Tcf-E2 activity was substantially below the wild type level in Co115 and Hct116 cells (Figure 2.7c & d, lanes 2, 7 & 12).

Since E1A13S is the transactivating isoform, cotransfection experiments were performed in SW480 and Co115 to examine whether the inhibition observed previously was specific to E1A12S (Figure 2.8). The E1A13S protein was potentially more active than the E1A12S protein on every promoter tested, in both SW480 and Co115 cells. In SW480, the absolute activity of all the promoters was roughly identical in presence of E1A13S. In Co115, the absolute activity of both Tcf-E2 promoters remained about 10-fold lower than the activity of the wild-type E2 promoter in Co115, despite a smaller inhibitory effect of E1A13S compared to E1A12S. Thus, experiments with the E1A13S isoform confirm that the inhibitory effect of E1A is stronger in semi-permissive than in permissive cell lines.
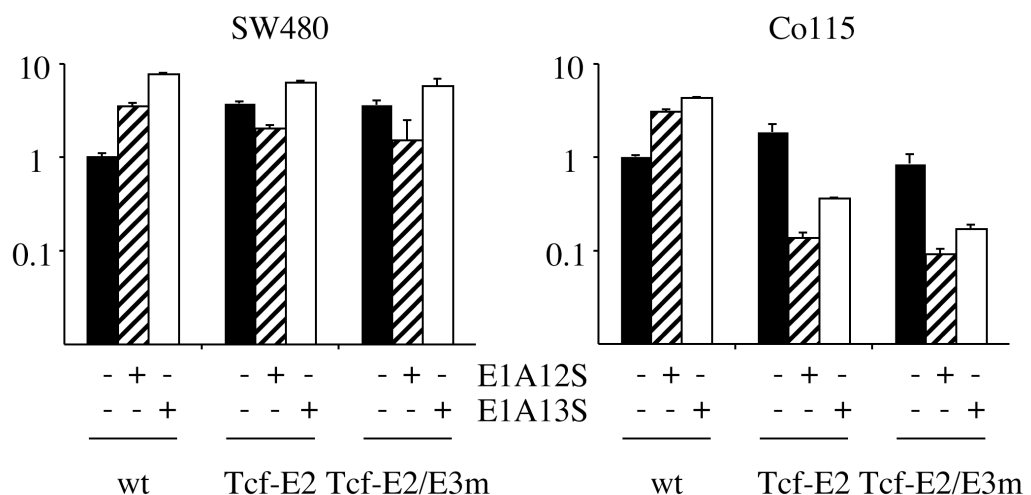

Figure 2.8: E1A-dependent inhibition is not relieved by E1A13S. SW480 or Co115 were transfected with luciferase reporters and plasmids expressing either the E1A12S or the E1A13S protein. The values are normalised to the value of the wild-type promoter in absence of E1A.

To determine the mechanism of inhibition, I tested different E1A mutants (Figure 2.7a). Mutation of the Rb binding site in E1A impaired transactivation of the wild type E2 promoter in SW480 and Co115 (Figure 2.7b & c, lane 3) but not in Hct116 cells (Figure 2.7d, lane 3), whereas mutation of the p300 or p400 binding sites had little effect on transactivation of the wild type promoter by E1A in all three cell lines (Figure 2.7b, c & d, lanes 4 & 5). Given the presence of E2F sites in the E2 promoter, one would expect reduced transactivation by an E1A mutant lacking its pRb-binding domain. The Tcf sites replace the normal enhancer in the Tcf-E2 promoter.[34] In all three cell lines the Rb and p400 binding site mutations did not relieve inhibition of the Tcf promoters by E1A (Figure 2.7b, c & d, lanes 8, 10, 13 & 15). A mutation in the p300 binding site (E1A Δ2-11, labelled Δp300N) partially relieved the inhibition, but in SW480 and Co115 the maximum recovery never exceeded 50% of the lost activity (Figure 2.7b & c, lanes 9 & 14). Mutation of E1A amino acid 2 to glycine (R2G), which also blocks p300 binding, had the same effect (data not shown). In Hct116, the Δp300N mutation completely restored activity of the Tcf-E2 promoter (Figure 2.7d, lane 9). Interestingly, I have noticed by western blot analysis that p300 is truncated in this cell line (the protein has a mobility on SDS-PAGE of ~240 kD, data not shown). Therefore, in Hct116 the effect of the mutation in E1A is surprising, but could reflect E1A binding to CBP or to the residual p300 fragment.

Analysis of additional E1A mutants

To investigate the cause of the incomplete recovery of Tcf-dependent transcription after mutation in the p300 binding site in E1A, additional luciferase assays were performed in H1299 cells (Figure 2.9). The Tcf-E1A promoter was activated 10-fold by ΔN-β-catenin (Figure 2.9a, compare lanes 1 & 2), and this activation was inhibited by E1A (Figure 2.9a, lane 3). p300 binds to two sites in E1A and mutation of either site partially relieved the inhibition of Tcf-dependent transcription (E1A Δp300N and Δp300C, Figure 2.9a, lanes 4 & 5). The C-terminal p300 binding site lies within conserved domain 1 (CR1), but deletion of the entire domain did not restore

activity (Figure 2.9a, lane 6). This suggests that there may be a positively acting factor which binds somewhere in CR1. To determine whether the E1A Δp300N mutation only partially restored activity because it did not completely block p300 binding, I cotransfected increasing amounts of p300 with E1A (Figure 2.9b). Exogenous p300 reversed the inhibition of promoter activity to the same extent as mutation of the p300 binding site (Figure 2.9b, lanes 4 & 7), and the effects of the Δp300N mutation and p300 transfection were not additive (Figure 2.9b, lane 8). This suggests that Δp300N completely blocks binding of E1A to p300. Large amounts of exogenous p300 reduced promoter activity (Figure 2.9b, lanes 5, 6, 9 & 10), suggesting that a cofactor was being titrated. P/CAF is a candidate for being this cofactor because it is a histone acetyltransferase (HAT) that binds to p300, and the coactivation of Tcf by p300 does not require intrinsic p300 HAT activity.[119] Since E1A inhibits P/CAF,[197] I tested whether mutation of the P/CAF binding domain in E1A relieved inhibition of Tcf activity by E1A and observed no effect (Figure 2.9a, lane 7). P/CAF was not limiting because cotransfection of P/CAF and wild type or ΔP/CAF mutant E1A also failed to restore activity (Figure 2.9c, lanes 4 & 9). To test whether p300 and P/CAF act together, I constructed an E1A gene with mutations in the binding sites for both HATs (labelled ΔΔ in Figure 2.9), but this mutant also failed to relieve the repressive effect of E1A (Figure 2.9a, lane 8). The same results were obtained when P/CAF was cotransfected with an E1A mutant in the p300 binding site (Figure 2.9c, lane 6), or when of p300 was cotransfected with an E1A mutant in the P/CAF binding site (Figure 2.9c, lane 8).

As in colon cells (Figure 2.7), mutation of the Rb binding site in E1A had no effect on repression of Tcf-dependent transcription (Figure 2.9a, lane 9). CtBP and TIP49 have both been implicated in transcription modulation by Tcf,[13,29] but neither mutations in E1A which abolish CtBP binding (ΔCtBP, ΔC52; Figure 2.9a, lanes 10 & 11) nor cotransfection of wild type or dominant negative TIP49 with E1A (Figure 2.9c, lanes 10 & 11) could overcome the repressive effect of E1A. In conclusion, the E1A mapping studies showed that mutation of the p300 binding domain could restore

about half of the Tcf activity lost upon E1A expression, but the remaining repressive effect could not be mapped to a known domain in E1A.
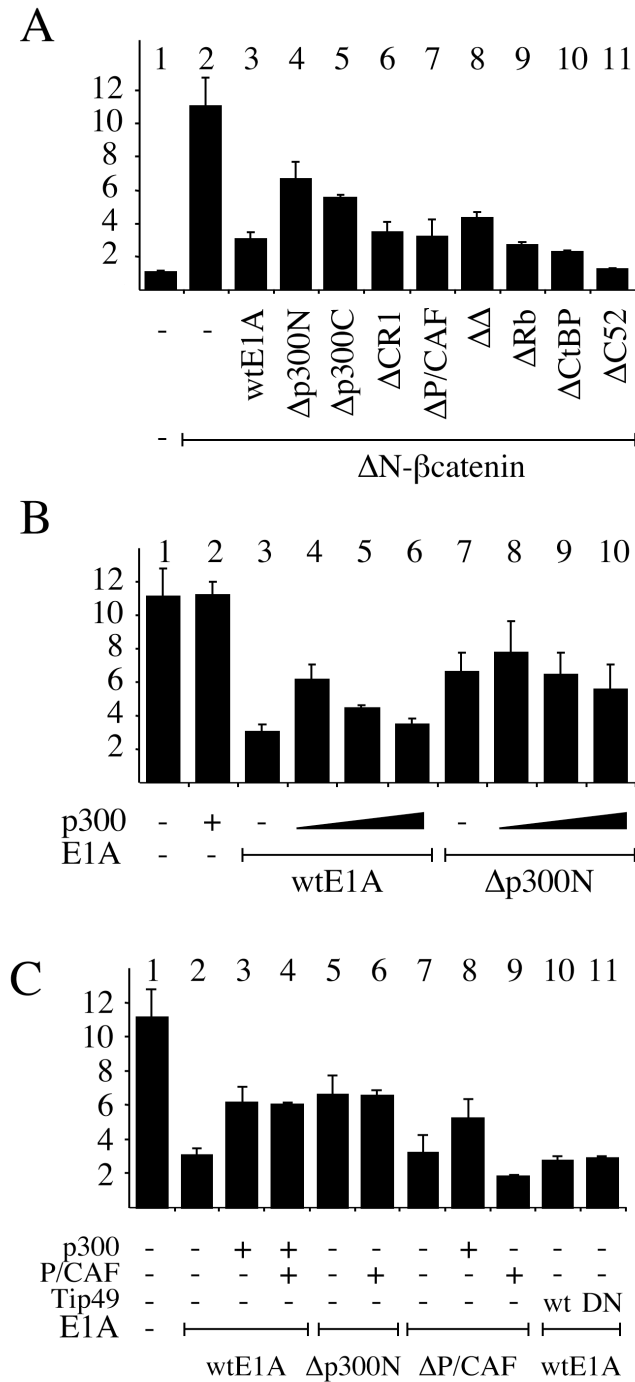

Figure 2.9: Luciferase assays in the lung cancer cell line H1299 showing inhibition of Tcf-dependent transcription by mutant forms of E1A. (A) Cotransfection of a Tcf-E1A reporter with various E1A mutants and ΔN-β-catenin. (B) Cotransfection of increasing amounts of p300 plasmid (0.5, 1, or 2 μg). (C) Effect of p300, P/CAF and Tip49 on Tcf-dependent transcription in the presence of wild-type and mutant forms of E1A. The values represent the fold activation versus the E1A wild-type reporter in the absence of E1A and ΔN-β-catenin on a linear scale.

E1A Δp300N mutant Tcf viruses

To test whether deletion of the p300 binding site in E1A would increase the activity of the Tcf promoters in the context of the virus, the Δp300N mutation was introduced into various Tcf-dependent backbones (See Figure 2.1 on page 40). For the Tcf-E1A promoter, inhibition of p300 by E1A should inhibit expression of E1A itself. This was tested by infecting the cMM1 cell line with A4 and AΔ4 (the Δp300N derivative of A4) in the presence and absence of tetracycline. Consistent with there being negative feedback by E1A on its own expression, the level of E1A after activation of Wnt signalling was higher with AΔ4 than A4 (Figure 2.4, compare lanes 7 & 8, E1A). Despite the increase in E1A expression, there was no difference in DBP expression, possibly because the Δp300N mutant is defective in some other function required for activation of the wild type E2 promoter (Figure 2.4, compare lanes 7 & 8, DBP). The viruses mutated at multiple sites were then tested on a panel of cell lines (Figure 2.5). The effect of the Δp300N mutation can best be appreciated by comparing matched pairs of viruses: A4 *vs*. AΔ4 (Figure 2.5, lanes 3 & 4); B23 *vs*. ΔB23 (Figure 2.5, lanes 9 & 8); and AB23'4 *vs*. AΔB234 (Figure 2.5, lanes 6 & 7). In each case the latter is derived from the former by deletion of the p300 binding site in E1A (the only exception is that the E3 promoter ATF site is present in AB23'4 but absent in AΔB234). In almost every case the Δp300N mutation actually reduced the level of expression of E1B55K, DBP and E4orf6. The only promoter whose activity was reasonably well maintained was the Tcf-E1A promoter (Figure 2.5, lanes 4 & 7, E1A). The wild type E1A promoter was also barely affected by the E1A Δp300N mutation (Figure 2.5, lane 8, E1A). The most comprehensively mutated virus (AΔB234, Figure 2.5, lane 7) was completely inactive in the control cell lines (H1299, HeLa and SAEC), but also 100-fold attenuated in the semi-permissive colon lines (Co115, HT29 and Hct116). When changes in multiple promoters were combined with the Δp300N mutation, the expression of DBP and E4orf6 even decreased in the permissive cell lines SW480 and ISREC-01, whereas in the absence of the Δp300N mutation, the virus with changes in multiple promoters (AB23'4) showed wild type

expression of E1A, E1B55K, DBP and E4orf6 in the same cell lines. The E1A Δp300N mutation did not increase E1B55K or DBP expression in any of the viruses with Tcf-E1B and Tcf-E2 promoters (Figure 2.5, compare lanes 6 *vs*. 7, and 9 *vs*. 8). Thus, in the context of the virus the E1A Δp300N mutation does not rescue the defect in Tcf promoter activity in the semi-permissive cell lines.

Stability of the E1AΔp300N protein

To determine whether the mutation in E1A had any effect on the stability of the protein, I performed a cycloheximide block on cells infected with either a wtE1A virus or with a Δp300N virus. The half-life of the E1AΔp300N protein was longer than that of the E1Awt protein (Figure 2.10). This probably results in higher E1A protein levels when the E1A protein is mutated to Δp300N. In the previous western blotting experiments, the activity of the E1A promoter was estimated by E1A protein levels, and might therefore have been overestimated because of the increased stability of the protein. If this is the case, it further indicates that the Δp300N mutation causes a reduction in viral activity and should be avoided in the development of Tcf-viruses.
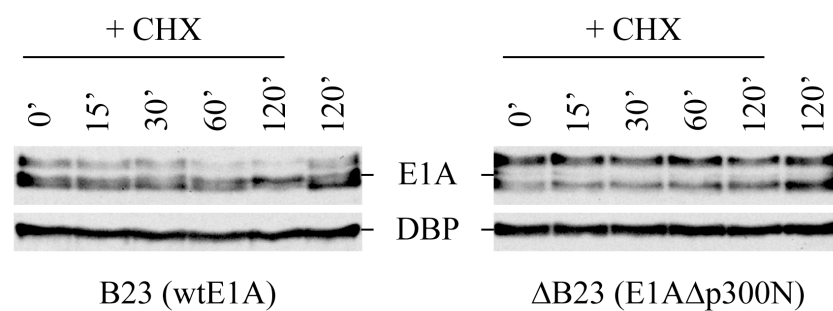

Figure 2.10: Cycloheximide (CHX) block showing the half-life of the E1Awt and E1AΔp300N proteins. Samples were taken at the indicated time after addition of 60 μg/mL of CHX.

Cytopathic effect of the Tcf-viruses

The new viruses were tested in cytopathic effect assays (CPE). In the most permissive colon cell line, SW480, both A4 and B23 were at least 10-fold more active than wild type Ad5 in CPE assays (Figure 2.11a, compare lane 1 with lanes 2 & 6). For these viruses, the corresponding p300 mutant viruses were about 10-fold less active (Figure 2.11a, compare lanes 2 *vs*. 3, and 6 *vs*. 7). Only for the AB23'4 virus was the p300 mutant virus as active as the parent (Figure 2.11a, compare lanes 4 *vs*. 5), but these viruses were 100-fold less active than the virus with only the Tcf-E1A/E4 changes (A4, Figure 2.11a, lane 2). In another permissive cell line, ISREC-01, A4 and B23 showed cytopathic effects comparable to wild type and AB23'4 activity was only 10-fold lower than wild type (Figure 2.11b). A4 showed wild type activity on Co115 (Figure 2.11c, compare lanes 1 *vs*. 2). This was 10-fold better than the previous best virus, B23 (Figure 2.11c, lane 6). In Hct116, the situation was reversed: B23 was slightly better than A4, but wild type was better than either Tcf virus (Figure 2.11d, lanes, 1, 2 & 6). In Co115, all of the p300 mutant viruses were 10-fold less active than the corresponding viruses with wild type E1A (Figure 2.11c, compare lanes 2 *vs*.. 3, 4 *vs*.. 5, and 6 *vs*. 7). In HT29, the least permissive colon cancer cell line tested, both the A4 and B23 viruses show 100-fold less activity than the wild-type virus and AB23'4 was further reduced by 10-fold (Figure 2.11e). All of the Tcf viruses were substantially less active than wild type Ad5 on HeLa cells, which lack Tcf activity (Figure 2.11f). The most engineered viruses failed to produce plaques on HeLa even after infection with 100 plaque-forming units (pfu) per cell (Figure 2.11f, lanes 4 & 5). The effect of the mutation of the p300 binding site in E1A was less obvious than on permissive cells. Overall, the best virus was A4, which was 10-fold less active than B23 and 1000-fold less active than wild type Ad5 on HeLa cells (Figure 2.11f, lanes 1, 2 & 6). Since A4 is 10-fold more active than wild type Ad5 on SW480, its overall selectivity for the most permissive colon cells is 10,000-fold relative to wild type Ad5.
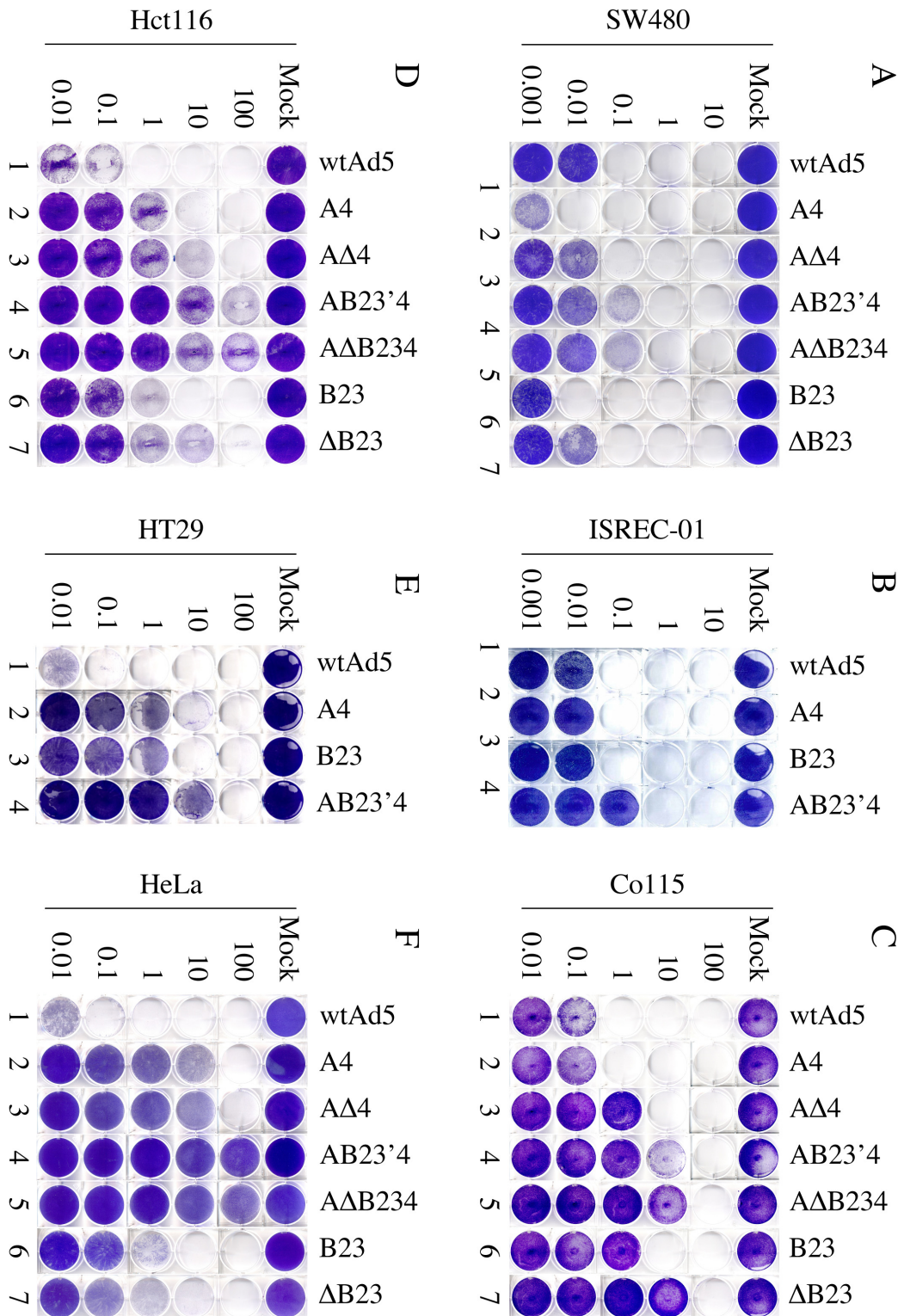
Figure 2.11: Cytopathic effect assays in different cell lines infected with 10-fold dilutions of wild type Ad5 and Tcf viruses. (A) SW480 cells were infected at a starting multiplicity of infection (MOI) of 10 pfu/cell and stained 6 days after infection. (B) ISREC-01 were infected at a starting MOI of 10 pfu/cell and stained 8 days after infection. (C) Co115, (D) Hct116, and (E) HT29 were infected at a starting MOI of 100 pfu/cell and stained 7 days after infection. (F) HeLa were infected at a starting MOI of 100 pfu/cell and stained 8 days after infection.

Burst assays of Tcf-viruses

Burst assays measure the amount of infectious viral particles produced during a single viral life cycle. In burst assays, the effect of the p300 binding site mutation was specific to the virus and the cell line. In SW480, the mutation reduced burst size 50-fold in the A4 backbone (Figure 2.12a, compare lanes 2 & 3), but had almost no effect in the B23 backbone (Figure 2.12a, compare lanes 4 & 5). This difference may be due to the fact that E2 promoter requires E1A function in AΔ4, where the wild type E2 enhancer is activated by ATF and E2F, but not in ΔB23, where the E2 enhancer is replaced by Tcf sites. The virus with Tcf sites in all the early promoters and the Δp300 mutation in E1A (AΔB234) was 100-fold less active than wild type in SW480, but almost as active as AΔ4 (Figure 2.12a, compare lanes 3 & 6). There was a striking reduction in AΔB234 burst size in the non-permissive cells (10^7-fold in HeLa cells, 10^5-fold in SAEC; Figure 2.12a, lanes 12 & 17). The remaining Tcf viruses showed 100 to 5000-fold reduction in burst size in HeLa and SAEC. The Δp300 mutation again reduced burst size in the virus with E2 driven by E1A (Figure 2.12a, compare lanes 8 & 9), but actually increased burst size (albeit from a very low level) in SAEC when the E2 promoter was driven by Tcf (Figure 2.12a, compare lanes 15 & 16). In general, the Δp300N mutation in E1A decreased viral burst size. This correlates with the reduction of the cytopathic effect shown previously (Figure 2.11). Viral bursts with selected viruses were further assayed in ISREC-01 and HT29 (Figure 2.12b). In both cell lines, the less mutated viruses (i.e. A4 and B23) were 5- to 10-fold less active than wild type, while the activity of AΔB234 was further decreased by about 10-fold. Finally, the A4 virus was analysed on a panel of cell lines, as well as human normal hepatocytes (nHNeps). The activity of the virus is shown in pfu produced per input pfu, which is the same as the burst size per cell if the multiplicity of infection is below 1 (Figure 2.12c). In colon cancer cell lines, the absolute burst size of the A4 virus varies between 100 and 1000 pfu/pfu. The absolute burst size is close to 1 in SAECs and hNHeps. This indicates that A4 is barely able to replicate if at all in normal cells. Figure 2.12d shows the relative burst size of A4 compared to wild type. In

all colon cancer cell lines tested, the burst size of A4 is not decreased by more than ~ 5-fold compared to wild type. On the other hand, it is approximately 100- to more than 1000-fold attenuated compared to the wild type virus in non colon cancer cell lines.
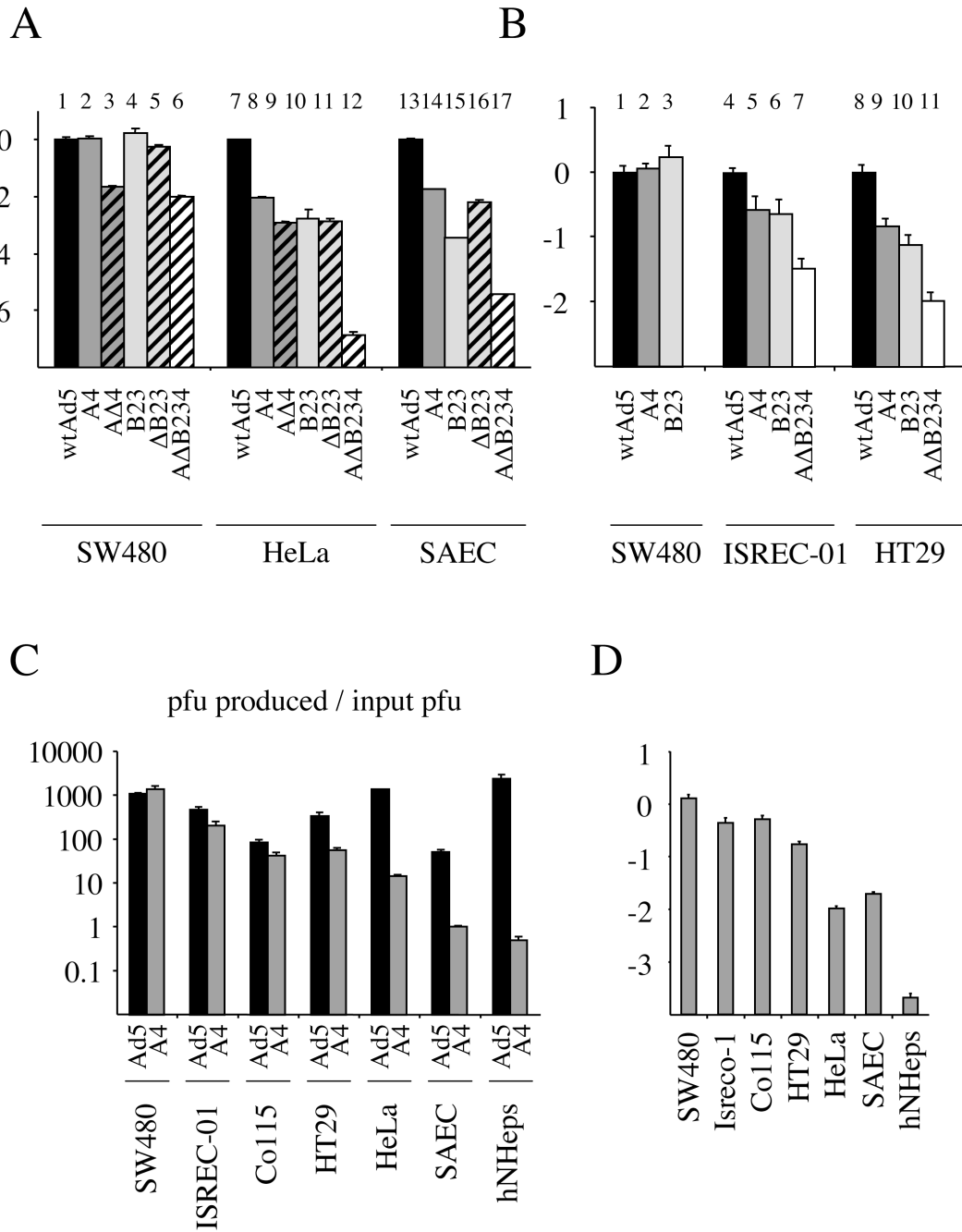
Figure 2.12: Viral burst assays on permissive and non-permissive cell lines. (A) SW480, HeLa and SAEC cells were infected with 300 viral particles/cell and lysed 48 hours after infection. The titre of viral particles present in the lysate was measured by plaque assay on SW480. Values were normalised to the wild type Ad5 titre on each cell line. (B) The same experiment was done on SW480, ISREC-01 and HT29. (C, D) A4 and Ad5wt viruses were tested on a broader panel of cell lines. Values are expressed either in pfu recovered per input pfu (C) or relative to Ad5wt (D). A, B, and D are in $\log_{10}$ scale.

2.3.3. Discussion

In this chapter, I have described adenoviruses that replicate efficiently in a wide range of colon cancer cells but not in normal cells. I tested viruses with Tcf sites in multiple viral early promoters and mutation of the p300 binding site in E1A. Compared to the previous generation of Tcf viruses,[34] A4 is less toxic to cells lacking Wnt activity and has broader activity in a panel of colon cancer cell lines in cytopathic effect assays. It has Tcf sites in both ITRs, but only E1A transcription is tightly regulated by Wnt signalling. This is partly explained by the fact that the Tcf sites are adjacent to the TATA box in the Tcf-E1A promoter, but several hundred base pairs upstream of the E4 TATA box. To create an E1A promoter with the minimum possibility of interference from extraneous signals, all of the normal regulatory elements were deleted in A4. This contrasts with the approach used to produce prostate, hepatocellular cancer and breast cancer targeting viruses, which retain the complete E1A enhancer but place exogenous promoters between it and the E1A start site.[102,125,201] To remove the E1A enhancer in A4 it was necessary to transfer the viral packaging signal to the right ITR. In addition, half of the ITR was replaced by Tcf sites. This construction dictated the position of the Tcf sites relative to the E4 start site. The endogenous E4 control elements were retained in A4 because they confer repression of E4 transcription in normal cells.[72] The mutant E4 promoter thus contains the part of the E1A enhancer contained in the packaging signal, which could activate the promoter, flanked by Tcf and E4F sites, which should repress the promoter in normal cells. The net result of these changes is reduced E4 transcription measured by luciferase assay, regardless of cell type.

Replication of the previous generation of viruses is restricted to cells with activated Wnt signalling by the Tcf sites in the E2 promoter.[34] AΔB234 and AB23'4, which have Tcf sites in multiple early promoters, were even more severely attenuated in cells lacking Wnt activity. In cytopathic effect assays in HeLa cells, they were at least 10^4-fold less active than wild type virus, and in burst assays in HeLa and SAEC, AΔB234 was 10^5 to 10^7-fold less active than wild type virus. The reduced activity of these viruses in

permissive cells might be due to deletion of element II in the E1A enhancer, which was previously reported to activate transcription of all of the early promoters in *cis*.[118] Comparison of different viruses shows that the Tcf-E1A and Tcf-E2 promoters display the same hierarchy of activity in a panel of colon cell lines, but relative to the corresponding wild type promoters, the Tcf-E1A promoter is more active than the Tcf-E2 promoter. This probably explains why A4 is more toxic than B23 in Co115 cells. The comparison between the different colon cancer lines is further explored in chapter 2.4.

Luciferase reporter assays showed a systematic inhibition of Tcf-dependent transcription by E1A. Mutagenesis of E1A indicated that this effect was partly due to inhibition of p300 by E1A, consistent with reports that p300 is a coactivator for β-catenin.[119,229] Coexpression of p300 together with E1A had the same effect on Tcf-dependent transcription as deletion of the p300 binding site in E1A, indicating that the remaining repression was unlikely to be due to inhibition of p300. The residual repressive effect of E1A could not be mapped to any known domain and merits further study. The negative results obtained with the ΔCR1 mutant are surprising because deletion of the CR1 p300-binding subdomain alone did partially restore Tcf-dependent transcription. This could conceivably be explained by an artefactual elevation of transcription of the renilla luciferase control by ΔCR1 E1A, but a more likely explanation is that another function of E1A is impaired by deletion of the entire CR1 domain.

The inhibition of Tcf-dependent transcription by E1A was greatest in the semi-permissive cell lines like Co115, resulting in very low luciferase activity because the starting level of Tcf activity was also lower in these cells. Hence, I expected to see a substantial effect of the Δ2-11 E1A mutation in the context of the viruses. In practice, the mutation produced no increase in expression from the Tcf-E2 promoter in colon cell lines and reduced the activity of the virus in cytopathic effect assays. There was a small but reproducible increase in E1A protein level in cMM1 cells expressing mutant β-catenin infected with AΔ4 virus compared to A4 virus. These results are consistent with decreased negative feedback of E1A on its own expression through relief of p300 inhibition, but the increase in E1A

level could be due to protein stabilisation. Consistent with the latter explanation, I have observed stabilisation of lower mobility E1A isoforms in SW480 infected with Δp300N virus. The mutation had complex and inconsistent effects in burst assays: it appeared to reduce burst size in permissive cells when the E2 promoter was driven by E1A (ie wild type), but increase burst size in some non-permissive cells when the E2 promoter was driven by Tcf. A general explanation is that any gain in Tcf activity due to the E1A mutation was offset by a loss of other E1A activities. In addition, there are some basal promoter activities regulated by E1A which may be abrogated by the Δ2-11 mutation.[142,156]

In conclusion, I have shown that adenovirus replication can be regulated by insertion of Tcf sites into the E1A or E2 promoters. Mutation of the p300 binding site in E1A did not increase transcription from Tcf promoters in the context of the virus. Since the E1A Δ2-11 mutation consistently reduced virus activity in cytopathic effect assays, it would be better to retain this domain in therapeutic viruses.

2.3.4. Materials and Methods

Adenovirus mutagenesis

An Ad5 E1A fragment (nucleotides nt 1 to 952) was amplified by PCR from ATCC VR5 adenovirus 5 genomic DNA with primers CGGAATTCAAGCTTAATTAACATCATCAATAATATACC (G76) and GGGTGGAAAGCCAGCCTCGTG (oCF1), cut with PacI, and cloned into the BamHI/PacI sites in pMB1 to give pCF4. pMB1 contains the left end of Ad5 cloned into the EcoRI/SmaI sites of pFL39.[24,34] The endogenous adenoviral sequence from the middle of the ITR to the E1A TATA box was replaced with four Tcf binding sites by inverse PCR with primers tccAGATCAAAGGGattaAGATCAAAGGGccaccacctcattat (oCF3) and tCCCTTTGATCTccaaCCCTTTGATCTagtcctatttatacccggtga (oCF4) to give pCF25 (the Tcf sites in the primers are shown in capitals). The final sequence of the mutant ITR and E1A promoter is catcatcaataatataccttattttgg attgaagccaatatgataatgaggTggtggCCCTTTGATCTTAATCCCTTTGATCTG

GATCCCTTTGATCTCCAACCCTTTGATCTAGTCC<u>tatttat</u>a, where the wt Ad5 sequence is in lowercase and the E1A TATA box is underlined. A G to T mutation was introduced just before the first Tcf binding site to mutate the Sp1 binding site.[152]

The Ad5 E4 fragment (nt 35369 to 35938) was amplified by PCR from VR5 DNA with primers G76 and ACCCGCAGGCGTAGAGACAAC (oCF2), cut with PacI and cloned into the BamHI/PacI sites in pMB1 to give pCF6. To compensate for the mutations introduced in the left ITR, three Tcf binding sites were introduced, and the endogenous sequence (nt 35805 to 35887) was simultaneously deleted by inverse PCR with primers oCF3 and tCCCTTTGATCTcc<u>actagt</u>gtgaattgtagttttcttaaaatg (oCF5) to give pCF16 (the Tcf site is shown in capitals and the SpeI site is underlined). The packaging signal was amplified by PCR from pCF6 with primers GAACTAGTAGTAAATTTGGGCGTAACC (oCF6) and ACGCTAGCAA AACACCTGGGCGAGT (oCF7), cut with SpeI/NheI and cloned into the SpeI site in pCF6 to give pCF34. The packaging signal has the same end-to-center orientation as at the left end of the adenoviral genome.

The Δ2-11 mutation was introduced in two steps. First, plasmids pCF4 (wild type E1A promoter) and pCF25 (Tcf-E1A mutant) were cut by SnaBI/SphI following by self-ligation to give pRDI-283 and pRDI-284, respectively. Second, the 2-11 region in pRDI-283 and pRDI-284 was deleted by inverse PCR with primers CATTTTCAGTCCCGGTGTCG (oCF8) and ACCGAAGAAATGGCCGCCAG (oCF9) to give pCF61 and pCF56, respectively.

The YAC/BAC vector pMB19[86] was cut with PacI followed by self-ligation to give pCF1, a YAC/BAC vector harbouring a unique PacI site.

In order to produce the gap repair vectors, combinations of left and right adenoviral ends were first assembled and then transferred to the YAC/BAC vector itself. During the first step, pCF34 was cut with EcoRI/Sal and cloned into the Pst/SalI sites of pCF25 to give pRDI-285. Similarly, pCF56 was cut with HindIII/SalI and cloned into the PstI/SalI sites of pCF34 to give pCF46. Finally pCF61 was cut with HindIII/SalI and cloned into the PstI/SalI sites of pCF16 to give pCF52. pRDI-285, pCF46

and pCF52 all contain a cassette with the left and right ends of the genome separated by a unique SalI site. These cassettes were isolated by PacI digestion and cloned into the PacI site of pCF1 to give pCF78, pCF79 and pCF81, respectively. pCF78 has mutant E1A and E4 promoters, pCF79 has mutant E1A and E4 promoters plus the Δ2-11 mutation, and pCF81 has wild-type E1A and E4 promoters plus the Δ2-11 mutation.

A4 (clone name: vCF011) and AB23'4 (clone name: vCF022) were constructed by gap repair[86] of pCF78 with Ad5wt (ATCC VR5) and B23' (clone name: vMB31)[34] DNA, respectively. AΔ4 (clone name: vCF042) and AΔB234 (clone name: vCF062) were constructed by gap repair of pCF79 with VR5 and B23 (clone name: vMB19)[34] DNA, respectively. ΔB23 (clone name: vCF081) was constructed by gap repair of pCF81 with B23 DNA. The viral DNA was cut with ClaI before gap repair to target the recombination event to a site internal to the mutations at the left end of the genome.

Viral genomic DNA was converted into virus by transfection of PacI digested YAC/BAC DNA into cR1 cells. The viruses were then plaque purified on SW480 cells, expanded on SW480, purified by CsCl banding, buffer exchanged using NAP25 columns into 1 M NaCl, 100 mM Tris-HCl pH 8.0, 10% glycerol and stored frozen at -70°C. The identity of each batch was checked by restriction digestion and automated fluorescent sequencing on a Licor 4200L sequencer in the E1A (nt 1-1050), E1B (nt 1300-2300), E2/E3 (nt 26700-27950) and E4 (nt 35250-35938) regions using primers IR213 (E1A antisense: CAGGTCCTCATATAGCAAAGC), IR190 (E1B sense: TGTCTGAACCTGAGCCTGAG), IR110 (E2/E3 sense: CATCTCT ACAGCCCATAC), IF171 (E2/E3 antisense: AGTTGCTCTGCCTCTCCA C) and IR215 (E4 sense: CGTGATTAAAAAGCACCACC). Apart from the desired mutations, no differences were found between the sequence of VR5 and the Tcf viruses. Particle counts were based on the OD_{260} of virus in 0.1% SDS using the formula 1 OD_{260} = 10^{12} particles/ml. Plaque-forming units (pfu) were estimated by counting plaques formed on SW480 cells after 10 days under 1% Bactoagar in DMEM 10% FCS.

E1A, p300, P/CAF, Tip49, β-catenin, hTcf4 and βcat-hTcf1 plasmids

Wild type 12S E1A (pCF9) and E1A mutants ΔpRb (124A,135A), Δp300N (Δ2-11), Δp300C (Δ64-68), Δp400 (Δ26-35), ΔP/CAF (E55), ΔCtBP (LDLA4), and ΔC52 were described by Alevizopoulos *et al*[4] and Reid *et al*.[197] All the mutants were provided in a pcDNA3 backbone (Invitrogen, Carlsbad, USA) except the Δp300N and Δp300C mutants that were isolated with BamHI/EcoRI and cloned into the BamHI/EcoRI sites of pcDNA3. The ΔCR1 mutant (Δ38-68) was made by inverse PCR of pCF9 with primers TCTGTAATGTTGGCGGTGCAGGAAG (oCF10) and ATGGCTAGGAGGTGGAAGAT (oCF12) to give pCF45. The ΔΔ p300-P/CAF double mutant was constructed by three-way ligation of BstXI fragments from the single mutants. The E1A12S mutants are schematised in Figure 2.7a. The E1A13s plasmid was constructed by digestion of pCF9 with BstXI (5.4, 0.68, and 0.32kb bands) and further digestion of the 0.68kb band by BsmBI to obtain a 0.54 kb band. pRc13s (R. Bernards) was cut with BstXI and BsmBI to purify the CR3 domain (0.27kb). A four-way ligation was done with the 5.4, 0.54, 0.32, and 0.27kb bands to get pCF113. This plasmid codes for E1A13S in a pcDNA3 backbone. The ΔN-β-catenin plasmid was described by van de Wetering *et al*.[243] The p300 vector contains HA-tagged p300 expressed from the CMV promoter. The P/CAF expression vector was described by Blanco *et al*.[23] The Tip49 and Tip49DN vectors were described by Wood *et al*[257]. The hTcf4 and βcat-hTcf1 were provided in a pcDNAI backbone by Nick Barker.[138]

Cell lines

ISREC-01,[37] SW480 (ATCC CCL-228) and Co115[55] were supplied by Dr B Sordat. HCT116 (CCL-247), HT29 (HTB-38), 293T were supplied by ATCC. HeLa (CCL-2) were supplied by ICRF. H1299 were supplied by Dr C Prives.[49] The cMM1 cells express myc-tagged ΔN-β-catenin[243] from the tet-off promoter.[164] C7 cells were supplied by Dr. J. Chamberlain.[5] To create the cR1 packaging cells, C7 cells were infected with a lentivirus expressing myc-tagged ΔN-β-catenin.[34] Clonetics small airway epithelial cells (SAEC)

and human normal hepatocytes (hNHep) were grown in SAGM and HCM media, respectively (Cambrex, East Rutherford, USA). All the other cell lines were grown in Dulbecco's Modified Eagle's Medium with 10% fetal calf serum (Invitrogen, Carlsbad, USA).

Luciferase assays

The E2 reporters were described by Brunori *et al.*[34] To construct E1A reporters, wild type and mutant E1A promoters were amplified by PCR from pCF4 and pCF25, respectively, with primers G76 and GTGTCGGAGCGGCTCGGAGG (oCF13), cut with HindIII, and cloned into the NcoI/HindIII sites of pGL3-Basic (Promega, Madison, USA). To isolate the E4wt promoter, pCF6 was cut with HindIII. The fragment containing the E4 promoter was isolated, cut with AluI, and inserted into the NcoI[Kf]/HindIII sites of pGL3-Basic (Promega, Madison, USA) to give pCV1. The Mut-E4 and Tcf-E4 reporters have been constructed using the same method with pCF16 and pCF34 to give pCV2 and pCV3, respectively.

Cells were seeded at 2.5×10^5 cells per 35-mm well 24 hours before transfection. 4.5 μl of Lipofectamine (Invitrogen, Carlsbad, USA) was mixed for 30 minutes with 100 ng of reporter plasmid, 1 ng of control Renilla luciferase plasmid (Promega, Madison, USA) and 500 ng of expression vectors. pcDNA3 empty vector was added to equalise the total amount of DNA. In Figure 2.9b, 0.5, 1 and 2 μg of p300 vector were used. Cells were harvested 48 hours after transfection and dual luciferase reporter assays performed according to the manufacturer's instructions (Promega, Madison, USA) using a Biocounter (Lumac bv, Landgraaf, The Netherlands). Each value is the mean of one to nine independent experiments done in triplicate and transfection efficiency is normalised to the activity of the Renilla control.

Western blotting

Cells were infected with 1000 viral particles per cell. Two hours after infection, the medium was replaced. Cells were harvested 24 hours later in SDS-PAGE sample buffer. Protein synthesis was blocked by addition of 60 μg/mL of Cycloheximide (Sigma, St Louis, USA). E1A, E1B55K, DBP and E4orf6 were detected with the M73 (Santa Cruz Biotechnology, Santa Cruz, USA), 2A6,[212] B6[196] and RSA3[167] monoclonal antibodies, respectively. Myc-tagged β-catenin was detected with the 9E10 monoclonal antibody.[69]

Cytopathic effect assay

Cells in six-well plates were infected with ten-fold log dilutions of virus. Two hours after infection, the medium was replaced. After six to eight days (Figure 2.11), the cells were fixed with paraformaldehyde and stained with crystal violet.

Virus replication assay

Cells in six-well plates were infected with 300 viral particles per cell. Two hours after infection, the medium was replaced. Cells were harvested 48 hours later and lysed by three cycles of freeze-thawing. The supernatant was tested for virus production by counting plaques formed on SW480 cells after 10 days under 1% Bactoagar in DMEM 10% FCS. Each bar in the figure represents the mean +/- SD of triplicate plaque assays.

2.4. Permissivity and viral replication

2.4.1. Introduction

As seen in the previous chapter and in work from other people, there is a clear overall trend for Tcf promoters to be less active in some colon cell lines.[34,47,155,206] The relative activity of a Tcf-promoter compared to that of a promoter with mutant Tcf sites can be considered as a measure of Wnt activity in a given cell line. In general, this value[33,206] correlates with the permissivity of the cells, as determined by their ability to sustain growth of Tcf-viruses. It is formally possible that the modest Tcf activity seen in some colon cell lines reflects a loss of activity that happened during tumour development or *in vitro* culture. In this case, there is no need to improve the existing Tcf viruses. On the other hand, this modest activity could be linked to specific genetic alterations found in these cancer cells. In this chapter, I will describe the experiments that were done to understand the semi-permissivity of these cell lines, and the approaches I took to circumvent that problem and make our viruses more active in this sub-population of colorectal cancer cells.

As seen in chapter 2.3.2, exogenous mutant β-catenin, hTcf4, or a fusion between the β-catenin transactivation domain and hTcf1, did not increase Tcf activity in reporter assays. Western blot analysis did not indicate any significant difference in total protein levels for β-catenin and hTcf4 between the cell lines (data not shown), suggesting that the levels of both proteins are probably not limiting. Therefore, the difference between permissivity and semi-permissivity should lie downstream of the Tcf/β-catenin complex, or, alternatively, at the level of β-catenin localisation.

BCL9 and Pygopus are likely candidates for being limiting factors in semi-permissive cell lines because they have been described as important effectors of the Wnt signalling pathway and have been shown to act downstream of the Tcf/β-catenin complex.[140] Transient transfection or lentiviral expression of these proteins however indicated that they are probably not limiting in semi-permissive cells. Interestingly, the subcellular

localisation of β-catenin was closely related to the permissivity of the cell line and correlated with the genetic defect present in the cell. Taken together, these observations indicate that the nature of the mutation in the Wnt pathway might predict the responsiveness of a given cell line to the Tcf-viruses.

The most cell type-sensitive Tcf-promoter appears to be E2 (see chapter 2.3). Time-course analysis of viral infections further confirmed this observation and indicated that the Tcf-viruses were less prone to enter the late phase of infection in the semi-permissive cell lines. Basal transcription factors such as TBP and TFIIB recognise the TATA and BRE elements, respectively.[103] Both of these elements poorly match their consensus sequences in the E2 promoter. In order to improve these sequences, I modified the spacing of the different elements, and inserted binding sites for known transcription factors such as E2F or FoxM1. E2F-dependant transcription is activated in S-phase and by the E1A protein.[225] FoxM1 is a forkhead transcription factor expressed in the area of the crypt where the Wnt pathway[54] is active, it is a marker for proliferating cells,[249] and is an interesting candidate for being a selector[99] factor for Wnt. Mutating the E2 promoter did not improve its activity. To have a more biologically relevant assay, I developed a new vector that makes it possible to test various promoters in the context of a viral infection. Finally, I will describe the analysis of an E1A protein that is fused to the HD2 domain of BCL9, which is the domain responsible for the binding of BCL9 to β-catenin. The rationale for creating such a fusion protein is to bring E1A to the vicinity of the early viral promoters through β-catenin-E1A interaction. The E1A fusion protein was able to activate transcription from the E2-Tcf promoter better than the wild type E1A protein, and this activation was dependent on an active Wnt pathway. I inserted the E1A-HD2 fusion protein into the AB23'4 background in order to test its ability to increase viral activity in the context of a virus.

2.4.2. Results

Expression of BCL9 and Pygopus1/2 proteins in CRC cells

BCL9, Pygopus 1 (Pygo1), and Pygopus 2 (Pygo2) are likely candidates for being limiting factors in semi-permissive cell lines because they have been described as important downstream effectors of the Wnt signalling pathway. BCL9 acts by bridging β-catenin to Pygo1/2, while the NHD domain of Pygo1/2 transactivates the promoters.[140] In order to check whether these proteins were limiting in the semi-permissive cell lines, their mRNA levels were measured by reverse transcription coupled to quantitative PCR (RT-qPCR) (Figure 2.13a). In general, there was no trend between mRNA levels and permissivity. Pygo1 was only detectable in HeLa, and BCL9 levels were 2-fold lower in CRC cells than in HeLa. Transient transfection experiments were performed to determine whether BCL9, Pygo1, or Pygo2 are limiting for Wnt activity. A Tcf-E2 luciferase reporter plasmid was cotransfected with various combinations of these proteins in the permissive cell line SW480 and the semi-permissive cell line Co115 (Figure 2.13b&c, respectively). In SW480, none of these proteins was able to activate the Tcf-E2 promoter, whereas BCL9 had a very marginal activating effect in Co115. This suggests that BCL9 or Pygo1/2 are not limiting in these cell lines.

A

B

Pygo1	-	+	-	-	+	+	-	+	-
Pygo2	-	-	+	-	+	-	+	+	-
BCL9	-	-	-	+	-	+	+	+	-

Tcf-E2   min

C

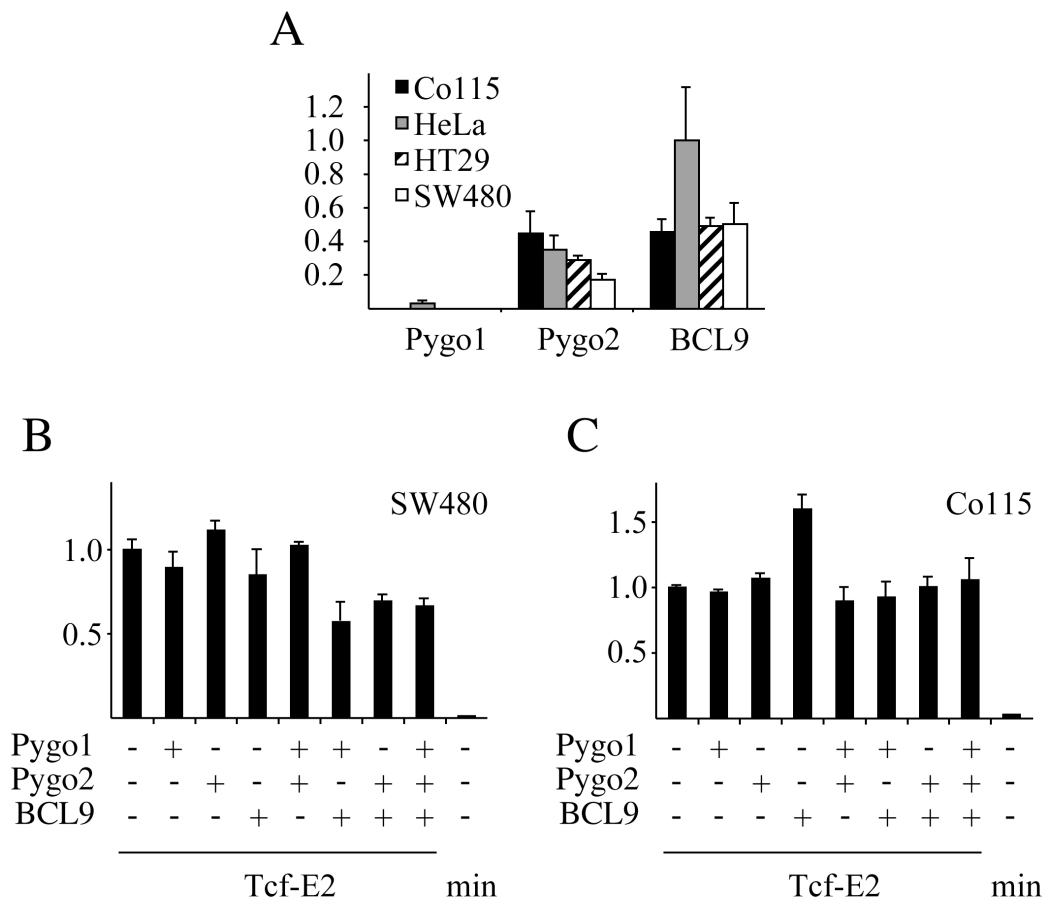|									
Pygo1	-	+	-	-	+	+	-	+	-
Pygo2	-	-	+	-	+	-	+	+	-
BCL9	-	-	-	+	-	+	+	+	-

Tcf-E2   min

Figure 2.13: (A) mRNA levels of Pygo1, Pygo2, and BCL9 were measured by RT-qPCR and normalised to 18s RNA. Transient transfection of Pygo1, Pygo2, and BCL9 in SW480 (B) or Co115 (C). Min is the E2 reporter in which all the activating sequences had been removed. Values are normalised to the activity of the Tcf-E2 promoter and represented on a linear scale.

To see if overexpression of these proteins could increase viral toxicity in the context of a viral infection, Pygo1 and Pygo2 were stably expressed from lentiviruses in SW480 and HT29 to produce the cell lines SW·P1, SW·P2, HT·P1, and HT·P2. After selection, overexpression of the Pygo proteins was confirmed by RT-qPCR (Figure 2.14a; "+"). Cytopathic effect assays were performed on these cell lines to determine whether this overexpression increased viral spread. As shown in Figure 2.14b, stable expression of Pygo1 or Pygo2 did not increase viral toxicity in either SW480 or HT29.

A

B
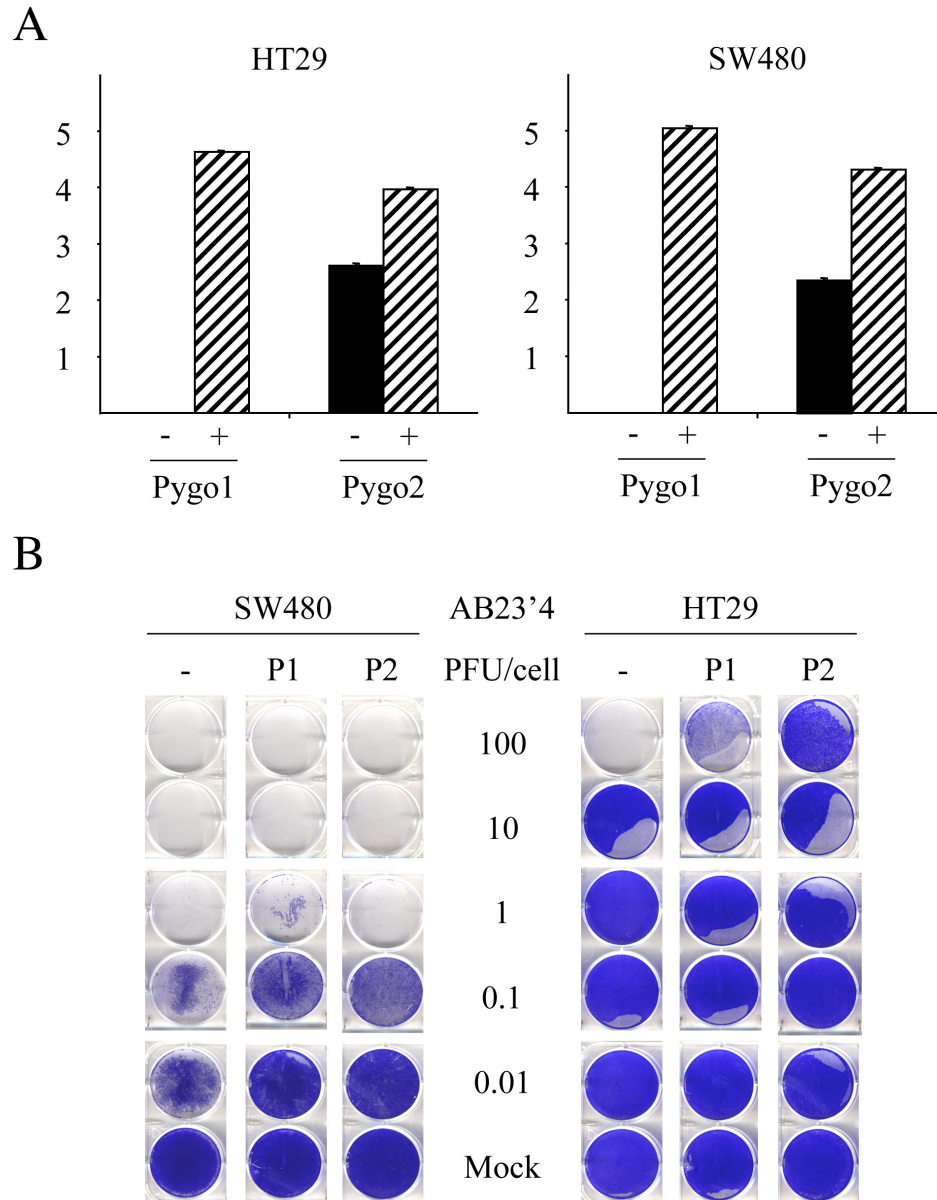
Figure 2.14: Cell lines stably expressing Pygo1 or Pygo2. (A) relative mRNA levels are expressed in arbitrary units. (B) Cytopathic effect assay in SW480 or HT29 cells, or their derivatives expressing either Pygo1 (P1) or Pygo2 (P2), with the AB23'4 virus. Cells were infected at a starting MOI of 100 pfu/cell and stained 6 days (SW480) or 9 days (HT29) after infection.

In conclusion, both transient and stable expression of BCL9, Pygo1 or Pygo2 showed that these proteins are not limiting in the semi-permissive cell lines Co115 and HT29.

Immunofluorescence assays

In the cell, β-catenin exists in three pools: membrane-bound, cytoplasmic, and nuclear. Wnt target genes are activated by nuclear β-catenin, while the other pools are unavailable for transcription. If the total amount of β-catenin is roughly equivalent between permissive and semi-permissive cell lines, the localisation of the protein might differ. Therefore, I used indirect immunofluorescence to determine the subcellular localisation of β-catenin in various cell lines. Non-permissive and semi-permissive cell lines showed only membrane staining but β-catenin was detected in the nucleus of the permissive cells (Figure 2.15). This indicates that the semi-permissivity might be due to mislocalisation of β-catenin rather than a missing downstream effector of the Tcf/β-catenin complex. The absence of β-catenin in the nucleus of the semi-permissive cells Hct116 and HT29 is incompatible with the modest, yet present, Wnt activity seen in these cell lines. It is possible that the level of β-catenin is below the detection limit in these cells. Alternatively, the epitope of β-catenin may be masked by other proteins *in vivo*.
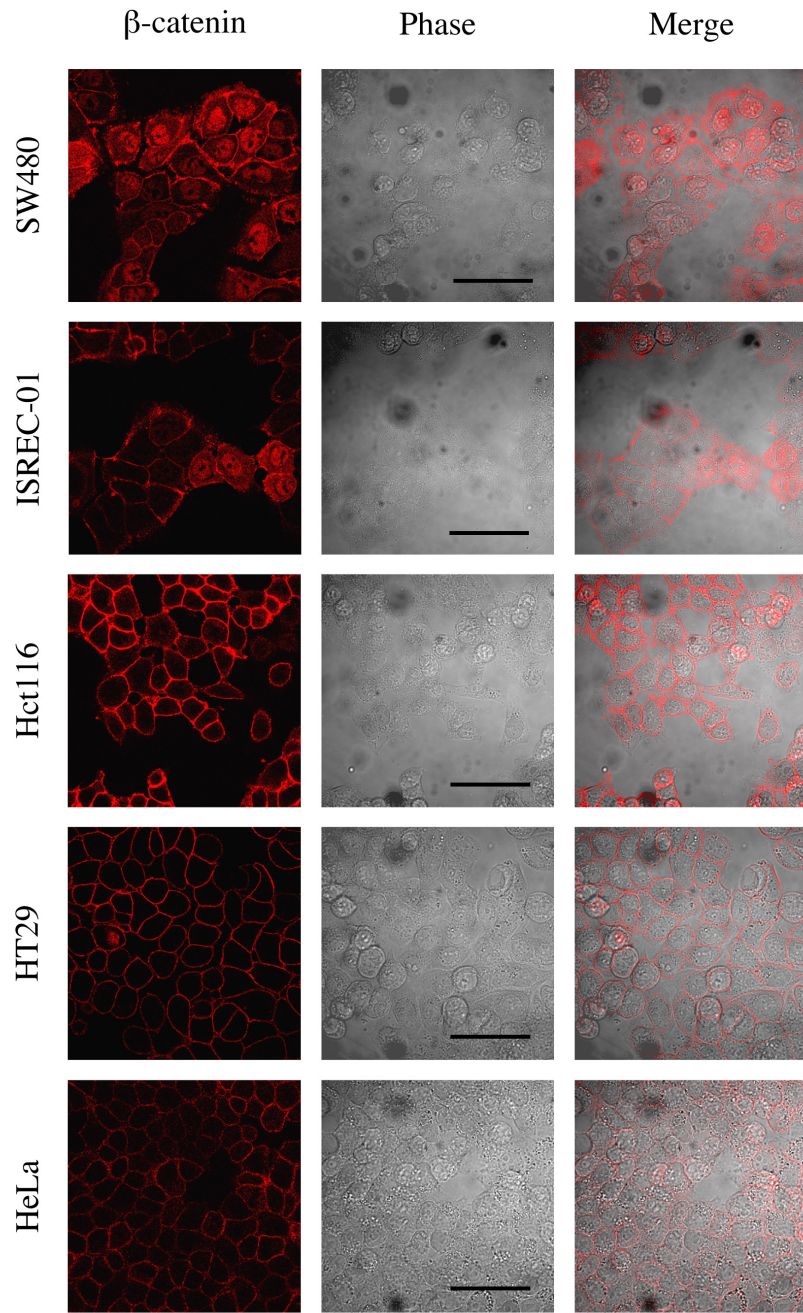
Bar = 50 μM

Figure 2.15: Detection of β-catenin by immunoflurescence assay. Images were taken using a confocal microscope to prevent background from the overlaying membranes.

Time-course expression of viral proteins

I showed in the previous chapter that the activity of the E2 promoter is the most sensitive to cell type. In order to determine which step of the viral life cycle is inhibited in the semi-permissive cell lines, time-course analysis of viral protein expression were done in SW480 and Co115. In SW480, all proteins were expressed similarly from the various viruses tested (Figure 2.16a). Fibre is expressed after viral DNA replication and was used as a marker for entry into the late phase of the viral life cycle. In SW480, the kinetics of fibre expression was comparable between all the viruses, and metabolic labelling experiments showed that viral late proteins were synthesised to a similar extent from all the viruses. Host cell proteins shut-off was obvious with Ad5wt- and B23-infected cells, whereas it was less pronounced when the cells were infected with the A4 and AB23'4 viruses (Figure 2.16b). Taken together, this confirms that the Tcf-viruses behave very similarly to the wild type virus in SW480.

Expression from the Tcf-E1A promoter was shown to be similar to that of the wild type promoter in Co115 (Figure 2.5, compare lanes 3&2). In contrast, expression from the Tcf-E2 promoter was drastically reduced compared to the native promoter (Figure 2.5, compare lanes 9&2). The pattern of expression of the E2 proteins DBP and pTP confirmed that the Tcf-E2 promoter is severely impaired in Co115 (Figure 2.17a). The fibre protein was not expressed from these viruses, suggesting that poor E2 protein expression resulted in reduced viral replication and slower progression to the late phase of the viral life cycle. In addition, no late viral protein was detected with the Tcf-E2 viruses in metabolic labelling experiments. Finally, the Tcf-E2 viruses induced less host cell protein shut-off than the wild-type virus (Figure 2.17b). All these experiments are consistent with a failure of the Tcf-E2 viruses to progress beyond viral replication during their viral life cycle, and the pattern of expression of the early viral proteins indicates that the most defective promoter in Co115 is the E2 promoter. In conclusion, the activity of the E2 promoter needs to be improved in order to render the Tcf-E2 viruses active in a broader panel of colon cancer cell lines.

Figure 2.16: The progression of the Tcf-viruses through the viral cycle is not impaired in SW480. (A) Western blot for E1A, E1B55K, DBP, E4orf6, E4orf6/7, and Fibre at various time points after infection with wtAd5 or Tcf-viruses. (B) Autoradiography. Cells were pulsed with ^{35}S-Methionine/Cysteine for one hour and collected at various time points after infection.
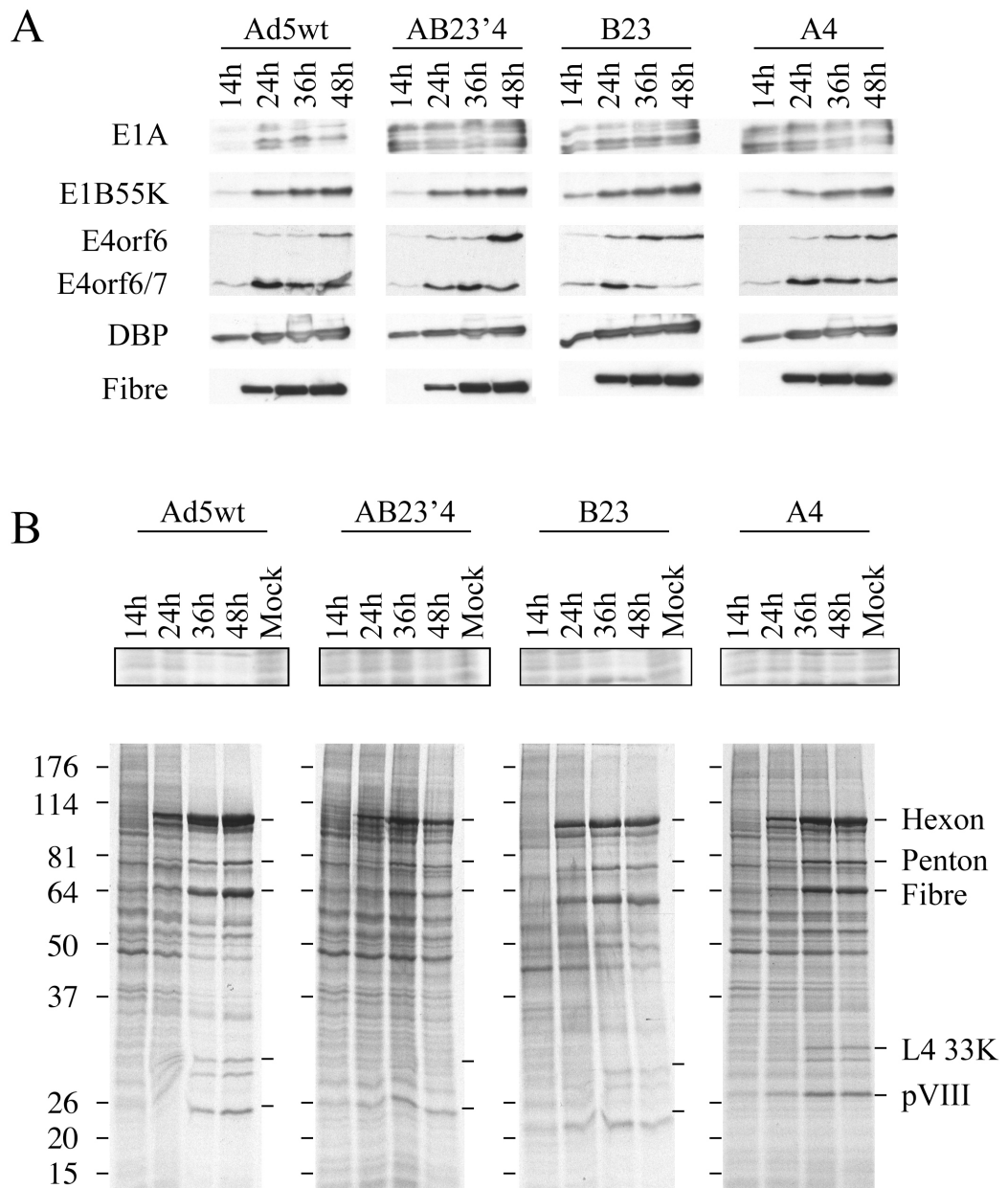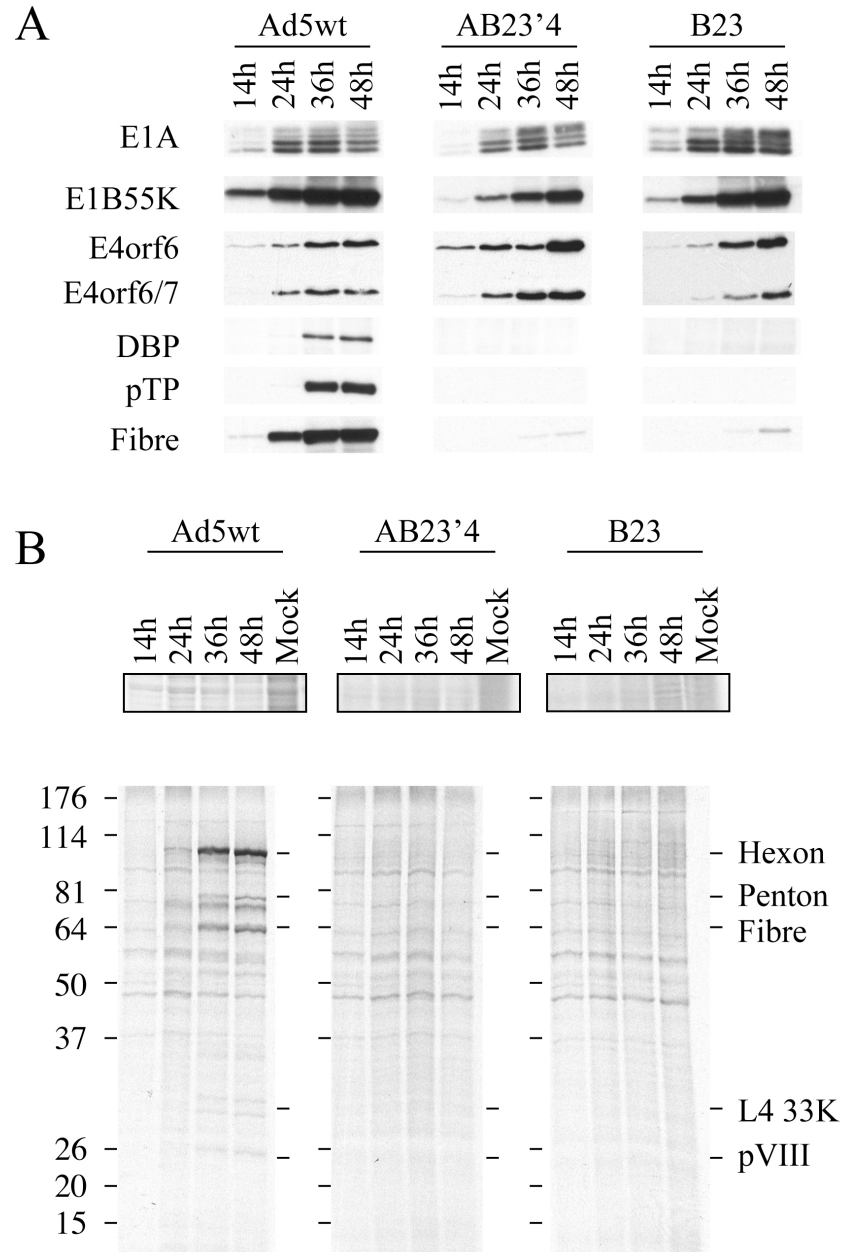
Figure 2.17: The progression of the Tcf-viruses through the viral cycle is impaired in Co115. (A) Western blot for E1A, E1B55K, DBP, pTP, E4orf6, E4orf6/7, and Fibre at various time points after infection with wtAd5 or Tcf-viruses. (B) Autoradiography. Cells were pulsed with ^{35}S-Methionine/Cysteine for one hour and collected at various time points after infection.

Mutagenesis of the E2 promoter

The structure of the wild type and Tcf-E2 promoters and sequences of the consensus binding sites for TBP (TATA) and TFIIB (BRE, TFII<u>B</u>-<u>r</u>esponsive <u>e</u>lement) are depicted in Figure 2.18. Both elements poorly match the consensus sequence and this might contribute to the poor activity of the Tcf-E2 promoter.

A

```
           < pVIII              ATF
...TTCCTTG CTCATAATGG CGCTGACGAC AGGTGCTGGC GCCGGGTGTG

              ATF         E2F                C/EBP     E2F
GCCGCTGGAG ATGACGTAGT TTTCGCGCTT AAATTTGAGA AAGGGCGCGA

          TATA                      CAP
AACTAGTCCT TAAGAGTCAG CGCGCAGTAT TTACTGAAGA GAGCC...
```

B

```
           < pVIII            Tcf              Tcf
...TTCCTTG CTCATAATGG CCCTTTGATC TTAATCCCTT TGATCTGGAT

     Tcf                 Tcf              TATA
CCCTTTGATC TCCAACCCTT TGATCTAGTC CTTAAGAGTC AGCGCGCAGT
```

C

Figure 2.18: Organisation of the wild type (A) and Tcf-E2 (B) promoters, described in the antisense orientation compared to viral situation. pVIII indicates the starting ATG of the pVIII protein, CAP is the E2 Cap site. (C) Comparison between the consensus and the actual BRE and TATA sequences, where BRE is the TFIIB response element. Bases that match the consensus are indicated in green, whereas bases that do not match the consensus are indicated in red. The underlined G in the BRE is essential for its proper function. S=C or G, R=G or A, W=T or A.
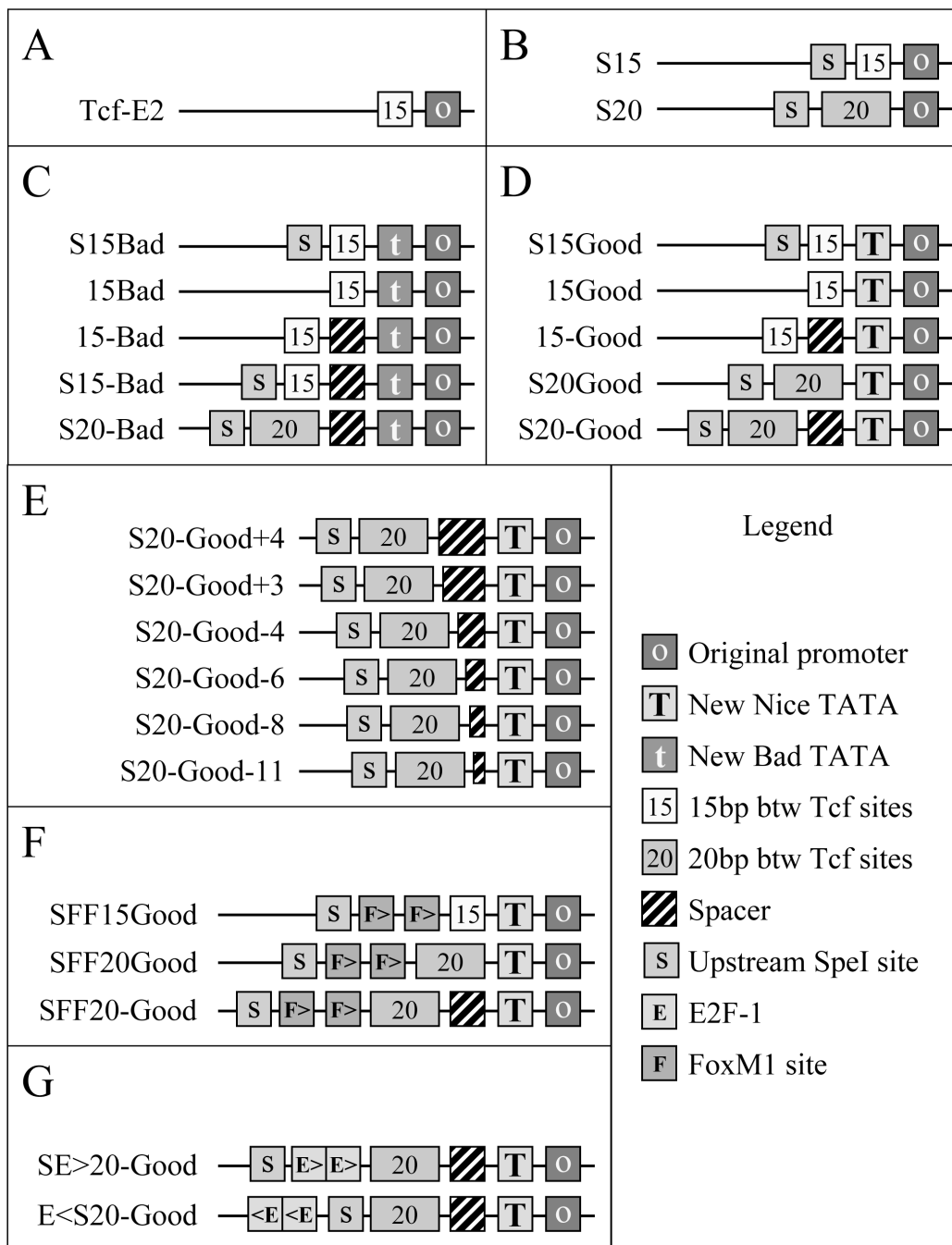
Figure 2.19: Structure of the mutant E2-Tcf promoters. (A) Tcf-E2 promoter used in the Tcf-E2 viruses. (B) 15bp- or 20bp-phased Tcf binding sites. (C) Duplicated native (bad) TATA box series. (D) Added (good) TATA box series. (E) Variation of the spacing between the first Tcf-binding site and the TATA box. (F) Addition of two FoxM1 binding sites. (G) Addition of two E2F binding sites. The name describes the promoter: 15=15bp-phased Tcf-binding sites; 20=20bp-phased Tcf-binding sites; S=SpeI site; Bad=addition of the native TATA box; Good=addition of an optimal TATA box; -=addition of a spacer between the Tcf sites and the TATA box; +4 to –11=addition or deletion in the spacer; FF=addition of two FoxM1 binding sites; E> or E<=addition of two E2F binding sites in the forward or reverse orientation.

I mutated the E2-Tcf promoter in order to increase its activity. The E2 TATA box could not be directly mutated because it contains the last codons of the 33K gene product (on the complementary strand). To circumvent this problem, I inserted a new optimal TATA box immediately upstream of the existing TATA. A non-optimal TATA box (i.e. the native E2 TATA box) was inserted at the same position as a control. The importance of the spacing between two Tcf binding sites was analysed by comparison of 15bp- and 20bp-spaced Tcf-binding sites. Finally, I constructed promoters with different length between the TATA box and the Tcf binding sites. These promoters are schematised in Figure 2.19.

In SW480, I showed by reporter assays that none of these modifications provoked any major difference in E2 promoter activity (Figure 2.20a). Rather surprisingly, insertion of an optimal TATA box did not result in higher transcription from the Tcf-E2 promoters. Similarly, varying the spacing between the first Tcf-binding site and the TATA box did not result in significant changes in the activity of the promoter (data not shown).

In Co115, some modifications decreased the activity of the promoter by approximately 4-fold, but no modification resulted in an important increase from the E2-promoter (Figure 2.20b). In addition, no difference in promoter activity was seen when the spacing between the TATA box and the Tcf sites was altered (data not shown). I conclude that none of these modifications can increase the activity of the E2 promoter.

Insertion of FoxM1-binding sites did not increase the activity of the E2 promoter, even when a plasmid coding for the FoxM1 protein was co-transfected (data not shown). Insertion of E2F-binding sites increased the activity of the E2 promoter by 25- to 30-fold in SW480, suggesting that an E2F activity is present in that cell line (Figure 2.21a). This is consistent with a study reporting inactivation of the p16 promoter in SW480.[87] In Co115, insertion of E2F-binding sites decreased the activity of the E2 promoter by approximately 50-fold (Figure 2.21b). Although this cell line was also reported to have inactivating mutations in the p16 promoter,[87] such results are compatible with E2F-mediated repression. Surprisingly, cotransfection of E1A (Figure 2.21; "+") did not affect E2 activity in either

cell line, suggesting that these activities are not modulated by E2F. In conclusion, none of the mutant promoters showed an increase in activity in both permissive and semi-permissive cell lines.
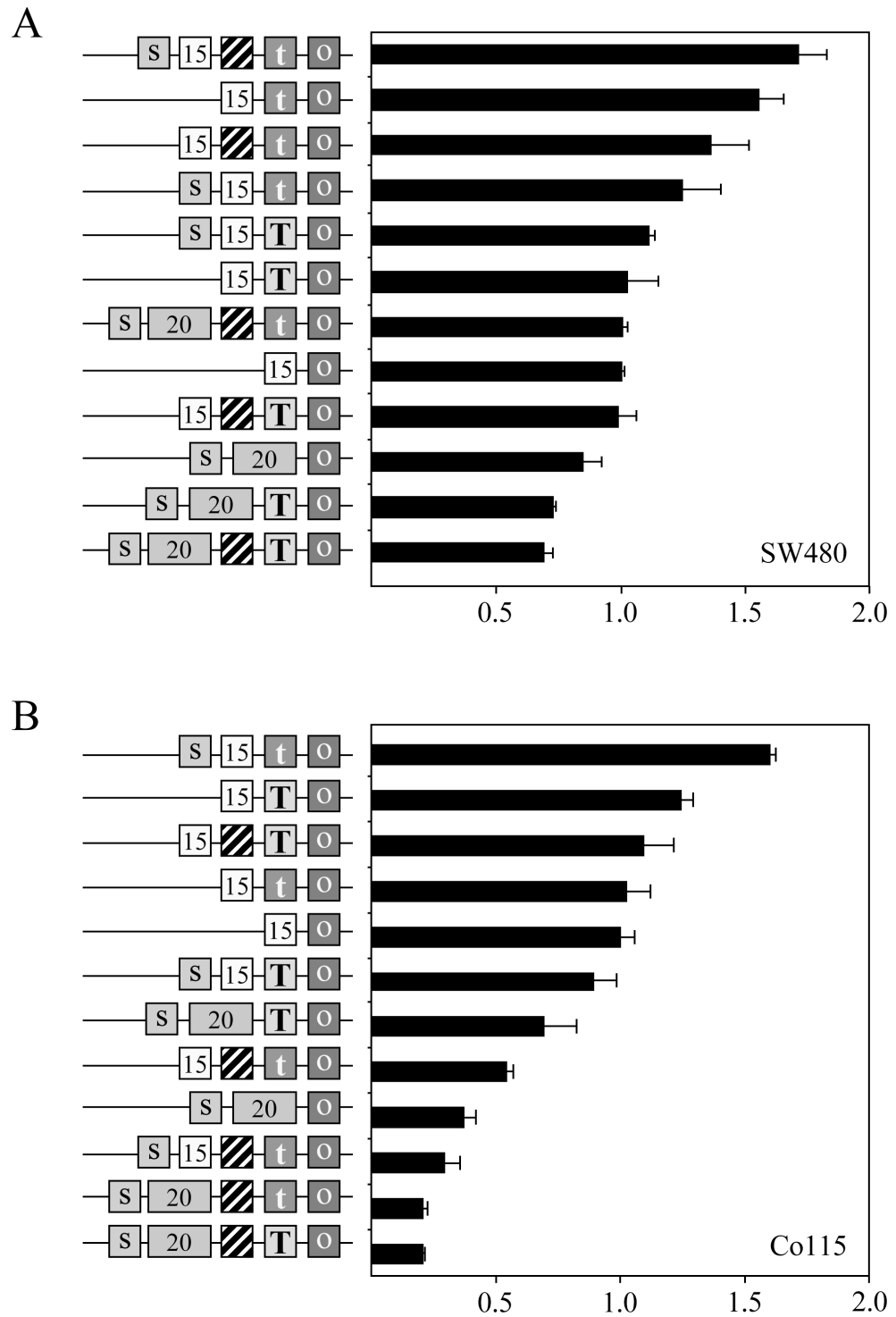
Figure 2.20: Activity of the new E2-Tcf promoters in SW480 (A) and Co115 (B). Values are normalised to the value of the E2-Tcf promoter used in the Tcf-viruses and plotted on a linear scale.
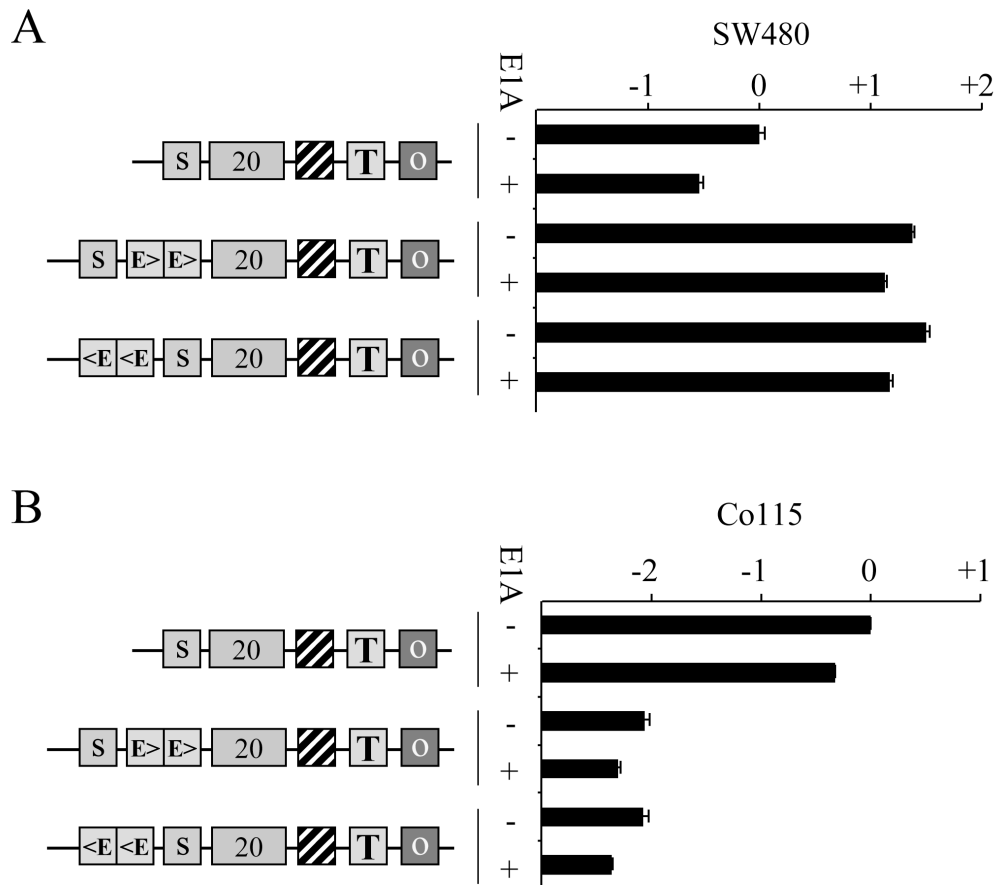
Figure 2.21: Activity of the E2-Tcf promoters containing E2F binding sites in SW480 (A) and Co115 (B). Values are normalised to the value of the E2-Tcf promoter used in the Tcf-viruses and expressed on a $\log_{10}$ scale.

Construction of a viral vector for rapid modification of the E2 promoter

It would be much more informative to be able to mutate the E2 promoter in the viral genome, and during a viral infection. I created a virus in which the E2 promoter is replaced by a tetracycline-inducible promoter and flanked by two different LoxP sites (Figure 2.22). This virus was created in an AB23'4 background to allow thigh selection of the E2 promoter and is called ABFT34, FT meaning Floxed- and Tetracycline-regulated E2 promoter. In principle, such a virus can grow normally in absence of tetracycline, provided that an activator like Tet-VP16 is present in the cell. In presence of tetracycline, promoter activation is abrogated and viral growth is inhibited. In Cre-expressing cells infected with ABFT34, a transfected E2 promoter sequence that is flanked by the two LoxP sites will

be incorporated in the viral genome by Cre-mediated recombination. Because these cells do not express Tet-VP16, only the recombined viruses will grow. Such a system could be used to incorporate a specific E2 promoter, or to select a promoter from a bank of mutant promoters.
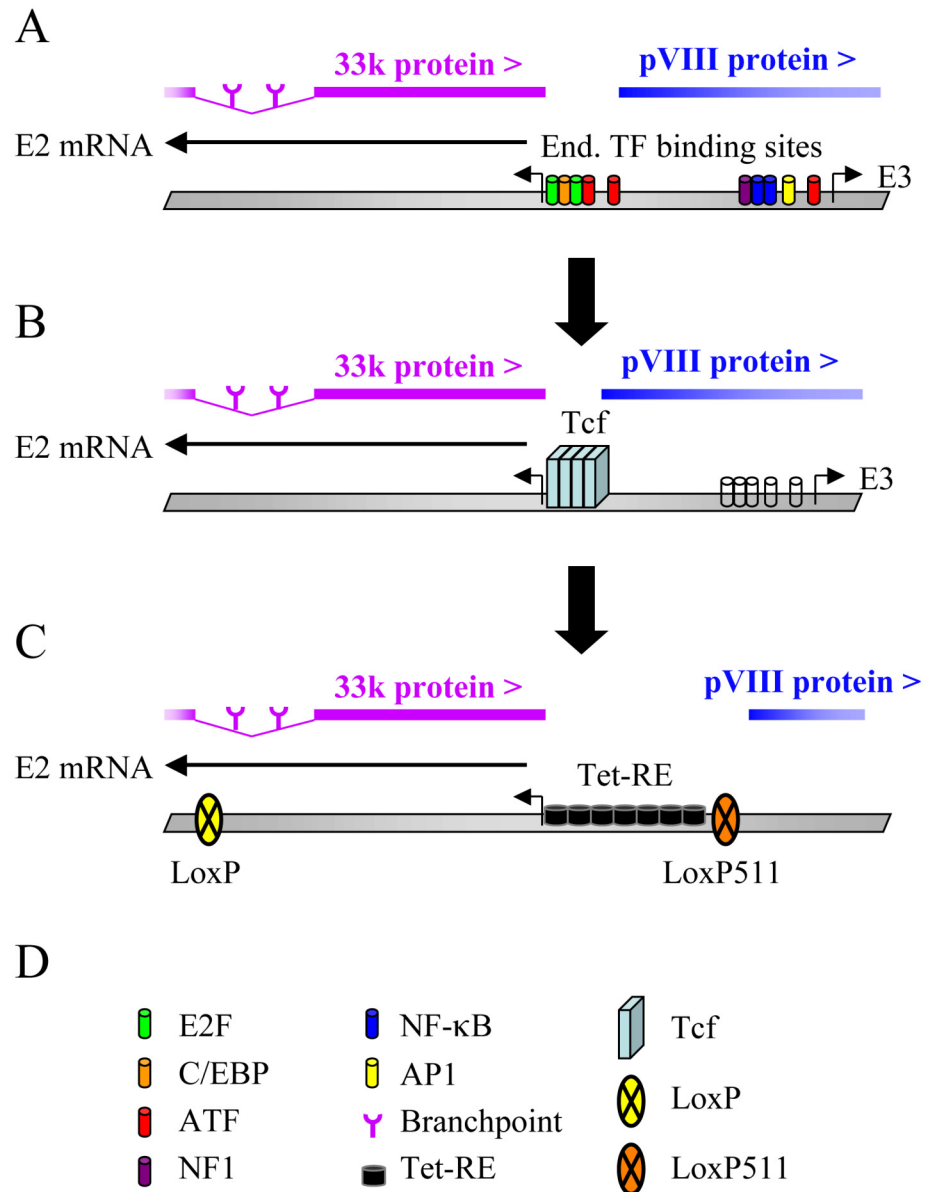
Figure 2.22: Schematic organisation of the E2/E3 region. (A) Wild type E2/E3 region with endogenous binding sites. The L4 33K mRNA (in pink) is composed of two exons (thick lines). In the intron (thin line), the two putative branchpoints are depicted. (B) Tcf-E2 region as present in the Tcf-E2 viruses. (C) Floxed-Tet-E2 region in ABFT34. The Tcf binding sites have been replaced by seven Tet-responsive elements. One LoxP site was introduced in the L4 33K intron and the other immediately upstream of the Tet-E2 promoter. (D) Legend.

Because the sequence coding for the late protein L4-33K on the rightward strand is embedded in the E2 promoter, it was not possible to

insert the first LoxP site immediately downstream of the E2 TATA box. To circumvent this problem, I inserted the first LoxP site in the intron of 33K, which is located further downstream of the E2 promoter. The second LoxP site, which is mutant (LoxP511), was inserted immediately upstream of the Tet-responsive elements of the E2 promoter (Figure 2.22).

To produce the virus containing the modifications mentioned above, the Tet-VP16 protein was stably expressed in CR1 and SW480 cells upon infection with a lentivirus coding for the Tet-VP16 transactivator. CR1·Tet-VP16 will be used for the initial conversion of plasmid DNA to viral particles and SW480·Tet-VP16 will be used to expand the virus. The Tet-VP16 activity of these cell lines was tested using a TET-E2 reporter plasmid (Figure 2.23). In both cell lines, activity of the TET-E2 promoter was comparable to that of the Tcf-E2 promoter (compare TET-E2 with Tcf-E2) but could be abrogated to nearly background levels (Min-E2) upon addition of tetracycline in the medium. Therefore, these cell lines appear suitable for production and expansion of ABFT34.

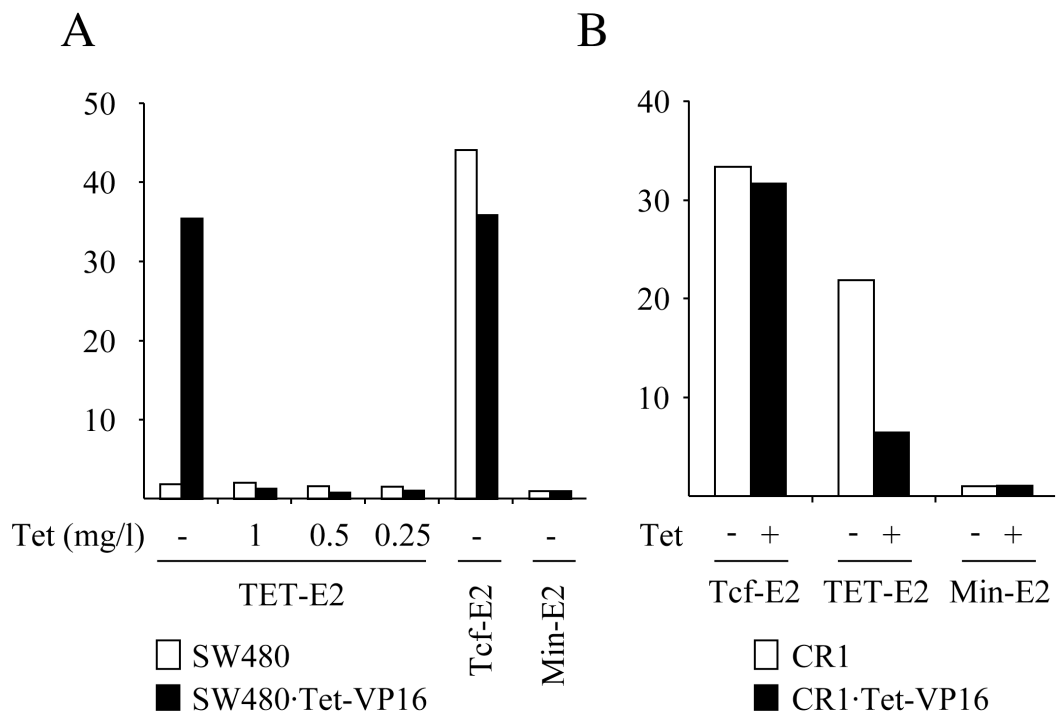Figure 2.23: Activity of the E2 promoters in SW480·Tet-VP16 (A) and CR1·Tet-VP16 (B). TET-E2=Floxed-TET-E2 promoter as depicted in Figure 2.22c; Tcf=Tcf-E2 promoter; Min=Min-E2 promoter. Values are normalised to the activity of the Min-E2 promoter and plotted on a linear scale.

Currently, the production of the ABFT34 virus has been unsuccessful. This could be explained by a possible interference between the LoxP sites and the expression of viral proteins. To bypass this problem, I propose to use a helper virus. Helper viruses are E1-deficient and can be propagated in complementing cell lines. Apart from E1, these viruses can provide all the other viral functions in *trans* and are used to produce viruses with deficiencies outside E1. The packaging signal of these viruses is flanked by two sites (i.e. LoxP or Frt) and can be excised by the corresponding recombinase (Cre or Flp, respectively), such that only the other virus is packaged. The ABFT34 virus might need the help of such a virus to be made or propagated.

E1A-HD2 fusion protein

TOPFLASH reporter assays and infection with Tcf-viruses suggest that the activity of the Wnt pathway is reduced in semi-permissive cell lines and that the E2 promoter is the most sensitive to the level of Tcf activity, and therefore to the cell type. It is possible that this promoter needs a higher threshold of activating signals. BCL9 binds to β-catenin through its HD2 domain in a way that does not prevent Tcf/β-catenin interaction. The E1A protein transactivates early viral promoters through interaction of its CR3 domain with ATF and the Mediator complex. In order to restore the transactivating potential of E1A, the HD2 domain of BCL9 was fused to the C-terminus of E1A. The fusion protein should bind to the Tcf/β-catenin complex and further activate transcription. To verify this hypothesis, the activity of the E1A12S-HD2 and E1A13S-HD2 proteins was tested by reporter assays.

In SW480, fusion of E1A to HD2 abrogated the E1A12S-dependent inhibition of the Tcf-E2 promoter (Figure 2.24a). The fusion protein was presumably still sequestering essential cellular proteins, though in the vicinity of the promoter. In a similar manner, transcription from the E2 promoter was better activated by E1A13S-HD2 than E1A13S (Figure 2.24a). This is compatible with the model that an E1A-HD2 fusion protein

that is tethered to the promoter can activate transcription. E1A13S probably activates the transcription through its CR3 domain.

In Co115, the HD2-proteins led to higher activity from the Tcf-E2 promoter that the parental proteins (Figure 2.24b). This is compatible with the model cited above. Tcf-dependent transcription was not inhibited by E1A in this set of experiments, so these results require confirmation.
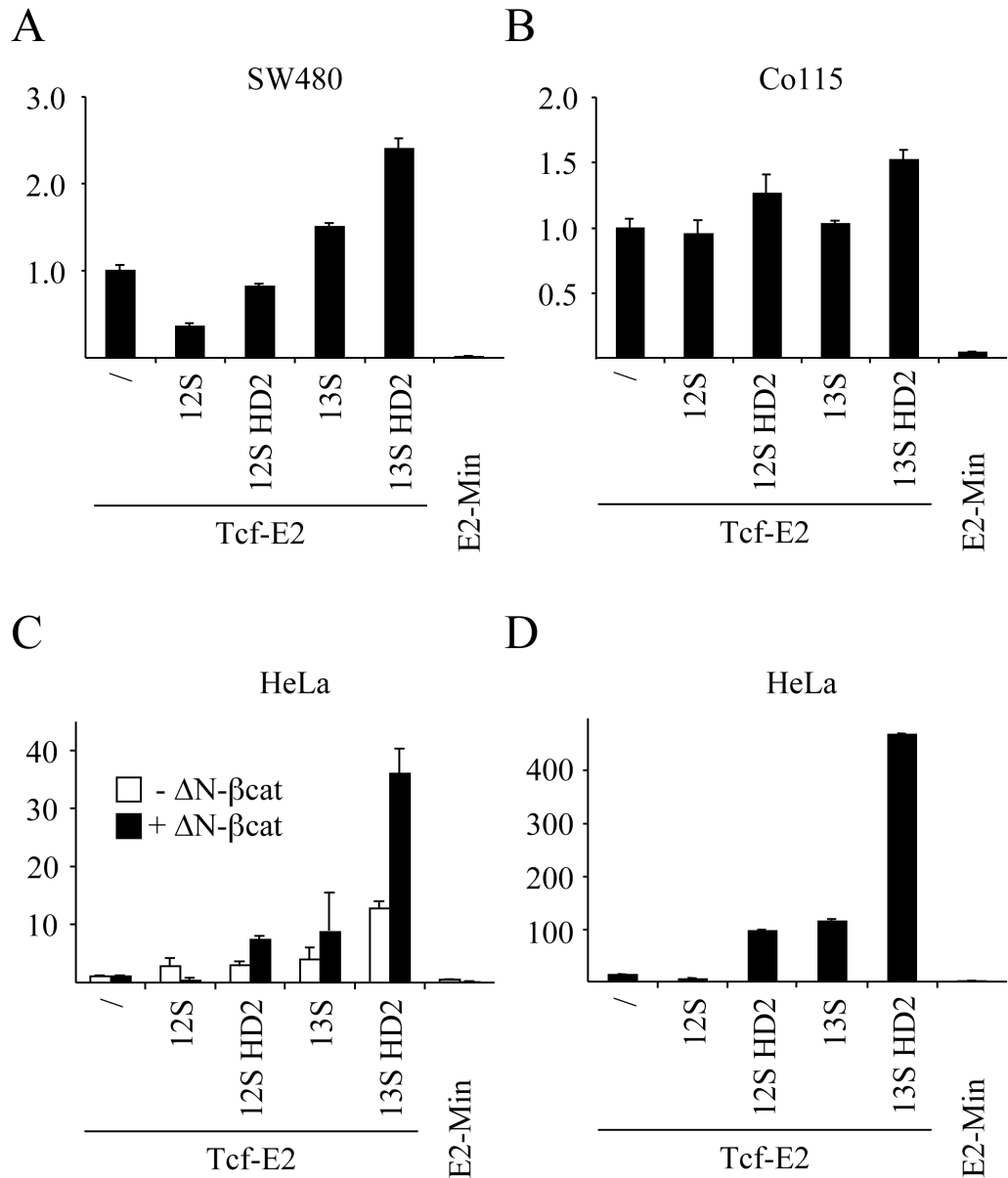
Figure 2.24: Reporter assays with E1A-HD2 proteins in SW480 (A), Co115 (B), and HeLa (C and D) cells. Values were not normalised to Renilla luciferase due to large variations of the activity of its promoter in presence of the E1A proteins. Values are normalised to the activity of the Tcf-E2 promoter in absence of any E1A protein (A to D). In (C), values are normalised within each group (+ or - ΔN-βcat), whereas in (D) all values are normalised to the activity of the Tcf-E2 promoter in absence ΔN-βcat.

In HeLa cells, the E1A13S-HD2 fusion protein activated transcription of the Tcf-E2 promoter by about 13-fold (Figure 2.24c), indicating either that HeLa have weak Wnt activity or that the fusion protein is somehow able to activate transcription independently of Wnt signalling. This activation was about 36-fold in presence of activated β-catenin (Figure 2.24c). In absolute values, E1A13S-HD2 and β-catenin together activate the transcription of the E2-promoter by about 470-fold (Figure 2.24d). This result indicates that the E1A-13S protein needs active Wnt signalling to efficiently activate transcription from a Tcf promoter. Thus, fusion of the HD2 domain of BCL9 to E1A in Tcf-viruses might increase expression from Tcf-promoters and improve their viral toxicity in colorectal cancer cell lines. The E1A gene was replaced by E1A-HD2 in an AB23'4 background to generate the AHB23'4 virus. This virus will enable testing the effect of E1A-HD2 during viral infection.

2.4.3. Discussion

In this chapter, I examined the differences between permissive and semi-permissive cell lines. As seen in the previous chapter, expression of components of the Tcf/β-catenin complex (or a fusion protein) could not increase the activity of a Tcf promoter in SW480 or in Co115 cells. This suggested that the block in semi-permissive cells either occurs downstream of β-catenin, or influences its level or localisation. I decided to look at the downstream effectors BCL9, Pygo1, and Pygo2, three proteins involved in Wnt/Wg signalling. In flies, homozygous mutations of these proteins cause embryonic lethality similar to that seen in *wg* mutant embryos. Moreover, Pygo1 had been reported to strongly activate Tcf-dependent transcription in HEK293 cells,[140] and inhibition of Pygo1 or Pygo2 by dsRNA has been shown to decrease the activity of a Tcf promoter in SW480 and Hct116 cells.[235] This indicates that BCL9/Pygo is a functional and important component of the Wnt signalling pathway.

No relevant difference in BCL9 or Pygo mRNA levels could be seen between various cell lines. Cotransfection of these proteins with a Tcf-E2 reporter did not result in increased expression of the reporter gene and the

toxicity of a Tcf-virus was not increased in cell lines that stably express Pygo1 or Pygo2. Taken together, these results suggest that these proteins are not limiting factors in the cell lines tested and thus cannot explain the difference between these two classes of cell lines.

I performed immunofluorescence assays on various cell lines and showed that non- and semi-permissivity correlated with the absence of β-catenin in the nucleus. In contrast, β-catenin was observed in the nucleus of SW480 and ISREC-01, two permissive cell lines. The widely accepted model is that β-catenin functions in the nucleus, where it binds to Tcf to activate transcription of target genes. Therefore, it is logical that permissivity should correlate with nuclear staining. The fact that no nuclear signal was seen in semi-permissive cells probably indicates that nuclear β-catenin levels are very low but sufficient to modestly activate transcription from Tcf promoters. Virtually all colon cancer cells have mutations in either APC or β-catenin. Most of the truncations in APC occur between the first and the third "20R" domains, in the so-called mutation cluster region (MCR, see Figure 1.5). These truncated APC proteins lose their ability to interact with axin, and thus to target β-catenin to the degradation complex. Interestingly, most of the cell lines permissive for Tcf-viruses or able strongly to transactivate in TOPflash assays contain truncations in the MCR (Type I truncations). In contrast, semi-permissive cell lines often contain mutations in β-catenin and retain a full-length APC protein. A puzzling exception was the HT29 cell line. This cell line is semi-permissive but contains a truncation mutation near the MCR. Nuclear export sequences have been reported either at the N-terminus or immediately after the third 20R domain of APC,[122,207] and the APC protein of HT29 retains both nuclear export sequences. Such truncations are called Type II truncations and have been associated with reduced Wnt activity.[206] Therefore, it is possible that the semi-permissivity is due to the presence of an APC protein that can export β-catenin to the cytoplasm. This would explain why Wnt activity could not be increased upon addition of β-catenin. A way to test this hypothesis is to silence the expression of APC by RNA interference (RNAi) and look at the localisation of β-catenin and at the activity of Tcf promoters.

Recent work in SW480 and Hct116 cells showed that APC RNAi could increase the activity of a Tcf promoter in Hct116 but not in SW480.[246] This further indicates that the "cytoplasmic export" theory might prove correct. If this is the case, our viruses should work in most colorectal cancers, since 85% of them have Type I APC mutations. Furthermore, most liver metastases show nuclear staining of β-catenin (K.J. Lipinski, *pers. comm.*).

Alternatively, detection of β-catenin in the semi-permissive cell lines might be impaired if the protein is complexed with cell type-specific inhibitors like chibby or ICAT, which are negative regulators of the Wnt pathway.[231,232] These proteins bind directly to a region between the last armadillo repeats and the C-terminus. Noticeably, this region is the one used to raise the β-catenin antibody. Thus, binding of these inhibitory factors might abrogate the detection of β-catenin in the nucleus of semi-permissive cells. Lastly, other pathways such as the TAK1/NLK/MAPK pathway have been shown to disrupt the Tcf/β-catenin complex and might play a role in the modulation of its activity.[130]

Analysis of viral protein expression showed that the E2 promoter is more sensitive than the E1A promoter to the cell type. Low expression of the E2 proteins presumably leads to reduced replication of the adenoviral DNA, and thus to a delayed late viral phase. In the semi-permissive cell line Co115, the Tcf-viruses failed to express E2 and late proteins. Analysis of the E2 promoter showed poor binding sites for proteins of the basal transcription machinery such as TBP and TFIIB. Nevertheless, improvement of these sites or addition of binding sites for other transcription factors did not result in an important increase in promoter activity. One can speculate that the non-optimal sequence of the E2 promoter ensures it is activated only after the expression of E1A and E4orf6/7 gene products. That further supports the hypothesis that the E2 promoter might need a higher "activating signal" than other promoters.

In order to increase this activating signal and recapitulate an E1A-induced activation of the E2 promoter, the β-catenin binding domain of BCL9 was fused to the C-terminus of E1A. Reporter assays showed that the fusion proteins are able to activate transcription from an E2 promoter better

than the native E1A proteins, and that proper activation depends on an activated Wnt pathway. Thus, E1A-HD2 might activate transcription from Tcf-containing promoters during the viral infection, leading to a Wnt-dependent, positive feedback loop. To test this hypothesis and to see whether expression of an E1A-HD2 protein is able to override the block seen in semi-permissive cell lines, I inserted the E1A-HD2 gene into the AB23'4 background. Such a mutation is compatible with viral growth and study of this virus might prove to be very interesting.

Because the E1A promoter is less sensitive to cell type than the E2 promoter and A4 is active in a broader panel of colon cancer cell lines, I suggest keeping the A4 background as long as the defect in the E2 promoter is not identified.

2.4.4. Material and Methods

Quantitative RT-PCR

Total RNA was extracted with the Qiagen RNeasy mini kit following the manufacturer's instructions (Qiagen, Hilden, Germany). Reverse transcription was performed with random hexanucleotides (Amersham Biosciences, Little Chalfont, UK) and Superscript II reverse transcriptase (Invitrogen, Carlsbad, USA). Quantitative PCR was performed on Roche Lightcycler using DNA Master SYBR Green I (Roche, Basel, Switzerland) following the manufacturer's instructions. Pygo1 was amplified with 200nM of primers oCF61 (TTCCTCGCATGGTGGTGA) and oCF62 (GAAAGAAGGTCCCTGTGTATTTG) at 62 °C. Pygo2 was amplified with 100nM of oCF63 (GGGCAAGGCCGGTCTG) and oCF64 (GTGAGT ATGCAGGGCCCTGAGTAT) at 62°C. BCL9 was amplified with 200nM of oCF65 (GATGGCCAATAAAGCTGCAG) and oCF66 (CTGTGTGTTC AGAGGCGCTG) at 63°C. The PCRs were performed in presence of 5 mM $MgCl_2$. The PerkinElmer pre-developed kit was used for 18S RNA detection (PerkinElmer, Wellesley, USA). Values are the result of three independent experiments.

HD2-E1A plasmids

E1A12S was isolated from pCF9 with BamHI and EcoRI and introduced into pUC19 to produce pALP1. A XmaI site was introduced at the C-terminus of E1A by iPCR on pALP1 with primers oCF83 (CGGGCCTGGGGCGTTTACAG) and oCF84 (GGTAAGGTGTAAACC TGTGATTGC) to produce pALP3. The region around the HD2 domain of BCL9 was amplified by PCR with primers oCF85 (TACCCGGGAGC AATAGCTCTTCAG) and oCF86 (ACCCCGGGATTCTGCTGCGGTCC CCCAC). The PCR product was cut by XmaI and inserted into pALP3 to give pALP5. The E1A coding sequence was cut from pALP5 with HpaI and BamHI and reintroduced into pCF9 to give pALP9. pALP9 has the E1A12S-HD2 gene in a pcDNA3 backbone (Invitrogen, Carlsbad, USA).

E1A13S was isolated from pCF113 with BamHI and EcoRI and introduced into pUC19 to produce pALP2. pALP5 was cut with PshAI and NdeI to isolate an E1A-HD2 fragment that was introduced into pALP2 to give pALP7. The E1A coding sequence was cut from pALP7 with HpaI and BamHI and reintroduced into pCF113 to give pALP8. pALP8 has the E1A13S-HD2 gene in a pcDNA3 backbone.

Lentiviral constructs

Plasmids coding for hPygo1 (pCF251), hPygo2 (pCF252), and BCL9 (pCF331) in a pBSIISK(-) backbone were kindly provided by K.Basler. pCF251 was cut with XhoI^{Kf} and EcoRI and inserted into pCF243 cut with SmaI and EcoRI to give pCF255. pCF252 was introduced similarly in pCF248 to give pCF256. pCF331 was cut with NotI^{Kf} and SalI$^{Kf(C/T)}$ into pCF247 cut with EcoRI^{Kf} and BamHI$^{Kf(G/A)}$ to give pCF343. The TET-VP16 sequence was isolated from pES126 (pBPSTR-1)[188] with Hind^{Kf} and BamHI and inserted into pCF243 cut with SmaI and BamHI to give pCF234. pCF243, pCF248, and pCF247 are pHR'-derived lentiviral backbones[176] that contain SV40 promoter-driven resistance genes against puromycin, neomycin, and phleomycin, respectively. In all these constructs, the transgene is driven by an EF1α promoter.

5x10^6 293T cells were seeded 24h before transfection in 10-cm dishes. One hour before transfection, medium was replaced with 4ml DMEM, 10% FCS supplemented with 25 μM chloroquine (Sigma, St. Louis, USA). Calcium-phosphate cotransfection was performed by mixing 6.5 μg packaging construct pCMV-ΔR8.91 and 3.5 μg pseudotyped envelope-encoding plasmid pMD2. VSVG (provided by D.Trono, Geneva) with 10 μg of lentiviral vector, in 250 μM final CaCl$_2$. An equal volume of 2xHBS (0.28 M NaCl, 0.05 M HEPES, 1.5 mM Na2HPO4, pH=7.10) was added. The precipitate was formed in 20' at room temperature and added to the 293T cells. Transfected cells were incubated 6 h at 37°C, then medium was replaced by 6 ml fresh DMEM, 10%FCS, and cells were incubated again at 37°C for 24 h. The viral supernatant was harvested with 8 μg/ml polybrene, filter-sterilized through a 0,20 μM pore filter, and frozen at –70°C before infection.

Production of stable cell lines and transfection assays

400'000 SW480 or 500'000 HT29 cells were seeded 24 hours before infection in a 35-mm dish. Cells were infected with 10x-, 30x-, and 90x-dilutions of lentiviral stocks overnight. Cells were passaged at 1/10 and 9/10 into 10-cm dishes. After one day, the medium was changed to medium containing 1 μg/ml puromycin or 1.6 mg/ml neomycin for selection of the integrants.

Luciferase Assays

Transfection and luciferase assays were done as described in chapter 2.3.4 using 0.5 μg of lentiviral plasmid to express the various proteins and 0.1 μg of Tcf-E2 promoter-driven luciferase as reporter.[34] The amount of DNA was normalised to 1 μg with empty lentiviral vector. Transfection efficacy was normalised using 5 ng of TK promoter-driven Renilla luciferase (Promega, Madison, USA). TET-E2 or E1A-HD2 transfections were done with Lipofectamine 2000 (Invitrogen, Carlsbad, USA), with a Lipofectamine 2000:DNA ratio was of 3:1 (v:w).

Immunofluorescence assay

30'000 to 100'000 cells were seeded onto a 15-mm glass coverslip (Fluka) into 24-well plates. Once the cells reached the desired confluence, wells were washed twice with PBS, and cells were fixed with $200\mu L$ formaldehyde 4% in PBS for 15' at RT. Cells were washed once with PBS and permeabilised with 0.1 % (v/v) Triton X-100, 1% (w/v) BSA in PBS for 10' at RT. Cells were washed three times with PBS and blocked with 5% BSA (w/v), 5% (v/v) FCS in PBS for 30' at RT. Cells were washed once with PBS and incubated with 200 μl of 250 μg/μl anti β-catenin antibody (C19220, BD Biosciences Pharmingen, San Diego, USA) in 0.5 % BSA, 0.5 % FCS in DMEM (staining buffer) at 37°C for 1h30'. Cy3-coupled anti-mouse secondary antibody solution (100 μg/ml) was centrifuged at 10'000 rpm at 4°C for 20' to pellet unconjugated Cy3. Cells were washed three times with PBS and incubated with 200 μl of 5 μg/ml centrifuged secondary antibody in staining buffer for 30' at 37°C. Cells were washed three times with PBS. The coverslips were mounted on slides in DABCO solution (Sigma, St-Louis, USA) and sealed with nail polish (Maybelline, NY, USA). DABCO solution is made by adding 245 mg of DABCO in 3 ml of PBS, adjusting the pH at 7.4, adding glycerol up to 9.5 ml and adding 0.5 ml of PBS 10X.

Western blotting

Western blotting was performed as described in chapter 2.3.4. Additional reagents were anti-fibre (RDI-ADENOV2abm, 1:2000, Research Diagnostics, Flanders, USA) and anti-pTP (IB6A8, 1:1000, gift of J.A. Engler)[76] antibodies.

Radiolabelling of proteins in infected cells

Cells in 35-mm dishes were infected with 1000 viral particles per cell for two days. The medium was replaced by 2 ml of DMEM (without L-methionine, L-cysteine, glutamine, sodium pyruvate, with Glutamax) containing 10 μCi of ^{35}S methionine (Redivue L-[^{35}S] Methionine or Promix

70% [35]S Methionine, 30% [35]S Cysteine, Amersham Biosciences, Little Chalfont, UK). One hour after addition of the radiolabelled amino acids, cells were collected in SDS-PAGE sample buffer, separated by SDS-PAGE, stained with Coomassie, dried for one hour on a DEAE membrane and developed on a Kodak Biomax MR film.

Mutagenesis of the E2 promoter

All the mutagenesis done on the E2 promoter was done starting from the pMB60 reporter, which contains the luciferase gene under the control of the Tcf-E2 promoter and has the nearby E3 promoter mutated.[34] Mutations were inserted by iPCR using primers oCF17 (new TATA box, CGACGCCT ATATATCTAGTCCTTAAGA), oCF18 (old TATA box, CTAGTCCTTA AGAGCTAGTCCTTAAGAGTCAGC), oCF19 (no spacer, ATCAAAGGG TTGGAGATCAA), oCF20 (spacer, CATATGGCTAGCTAAGCGATCAA AGGGTTGGAG), oCF21 (20bp spaced-Tcf, CATTGCCCTTTGATCTCC TACTGAACCCTTTGATCG), oCF22 (20bp spaced Tcf + SpeI, CATAAG ATCAAAGGGACTGTAGACAGATCAAAGGGACTAGTGCCATTATG AGCAAGG), oCF27 (15bp spaced Tcf + SpeI, AGTCCCTTTGATCTTAA TCCCTTT), and oCF28 (15bp spaced Tcf + SpeI AGTCCATTATGAGCA AGGAAATT). Various combinations of templates and primers led to pCF plasmids that were backcloned to give the corresponding pCV plasmids (Table 2.1).

FoxM1 binding sites were inserted by annealing oCF23 (CTAGTAC GTTGTTATTTGTTTTTTTCG) with oCF24 (CTAGCGAAAAAAACAA ATAACAACGTA) and inserting the double-stranded oligonucleotide into pCV20, pCV21, or pCV26 cut with SpeI. Similarly, E2F binding sites were inserted by annealing oCF35 (CTAGTTTTCGCGCCTGCGTTTTCG CGCG) with oCF36 (CTAGCGCGCGAAAACGCAGGCGCGAAAA) and inserting the double-stranded oligonucleotide into pCV21 or pCV26. Various combinations are summarised in Table 2.2.

Template	Oligo1	Oligo2	Plasmid	Backcloned	Name*
pCF21	oCF17	oCF19	pCF118	pCV13	15Good
pCF21	oCF18	oCF19	pCF122	pCV15	15Bad
pCF21	oCF17	oCF20	pCF127	pCV17	15-Good
pCF21	oCF18	oCF20	pCF128	pCV19	15-Bad
pCF21	oCF21	oCF22	pCF162	pCV25	S20
pCF21	oCF27	oCF28	pCF169	pCV29	S15
pCF118	oCF21	oCF22	pCF132	pCV20	S20Good
pCF118	oCF27	oCF28	pCF137	pCV21	S15Good
pCF122	oCF27	oCF28	pCF144	pCV23	S15Bad
pCF127	oCF21	oCF22	pCF164	pCV26	S20-Good
pCF128	oCF21	oCF22	pCF167	pCV27	S20-Bad
pCF128	oCF27	oCF28	pCF171	pCV30	S15-Bad

Table 2.1: generation of mutant E2 promoters in reporter plasmids. * 15=15bp between two Tcf sites, 20=20bp between two Tcf sites, S=SpeI site 5' of the Tcf sites, -=spacer between Tcf sites and TATA box, Good=insertion of a new optimal TATA box, Bad=insertion of a new native TATA box

Template	Sites	Product	Name*
pCV20	FoxM1	pCF174	SFF20Good
pCV20	FoxM1	pCF175	SF20Good
pCV21	FoxM1	pCF176	SFF15Good
pCV21	FoxM1	pCF177	SF15Good
pCV26	FoxM1	pCF180	SFF20-Good
pCV26	FoxM1	pCF181	SF20-Good
pCV26	E2F	pCF186	SE>20-Good
pCV26	E2F	pCF188	E<S20-Good
pCV21	E2F	pCF189	SE>15Good

Table 2.2: Generation of FoxM1- and E2F-binding sites-containing E2 promoters. * as in Table 2.1, additional symbols: F=FoxM1 binding site, E>=2 E2F binding sites in the forward orientation, <E=2 E2F binding sites in the reverse orientation

Changing the spacing between the Tcf sites and the TATA box was done from pCV26, where spacer sequence was cleaved with NheI, NdeI, or BlpI. The ends were processed with either the Klenow fragment or the Mung Bean nuclease. The plasmid was then re-ligated and gave pCF193 (-6 bp compared to pCV26), pCF194 (-4bp), pCF195 (+4 bp), pCF196 (+3 bp), pCF197 (-8bp), and pCF199 (-11 bp).

The TET-E2 promoter was isolated from pCF249 (see below) cut with EcoRI and AflII and inserted into pMB39[34] cut with EcoRI and AflII to give pCF271.

Floxed-Adenovirus mutagenesis

A LoxP511[239] site was introduced before the E2 promoter by iPCR of the plasmid pMB66 (the integrating vector with mutant E2 and E3 promoters[34]) with primers oCF46 (ATACTATACGAAGTTATCGCCATT ATGAGCAAGG) and oCF47 (ACATTATACGAAGTTATCTCGAGTTA ACCTAGTCCTTAAGAGTCA) to yield pCF237. The Tet-responsive element was isolated from pES149 (pUHD10-3)[95] cut with StuI and XhoI and inserted in the E2 promoter of pCF237 cut with HpaI and XhoI to give pCF249. A LoxP site was introduced into the 33K intron by iPCR of pCF157 with primers oCF48 (ATGCTATACGAAGTTATTTAGCCCAAG AGCAACA) and oCF49 (ACATTATACGAAGTTATCGGCGGCGGCTG CTTGG) to yield pCF241. pCF157 is the KpnI fragment of Ad5 (nt 25838 to 28592) in pUC19. pCF241 was cut with KpnI[T4] and AflII and inserted into pCF249 cut with SgrAI[Kf] and AflII to give pCF253, the integrating vector with the floxed TET-E2 promoter. The floxed TET-E2 sequence was introduced into an AB23'4 backbone using two-step gene replacement in yeast as described previously[86] to give ABFT34 (FT standing for Floxed- and TET-E2). Viral genomic DNA was converted into virus by transfection of PacI digested YAC/BAC DNA into cR1 cells. The viruses were then purified, expanded and characterised as described in chapter 2.3.4.

HD2-Adenovirus mutagenesis

A SalI site was inserted into the multiple cloning site of pcDNA3 by cloning the HindIII/EcoRI fragment of pUC19 into pcDNA3 to give pUCDNA3. vpCF2 is the AB23'4 genomic sequence in a YAC-BAC vector. The leftmost part of vpCF2 (nt 1 to 1748) was cut with SpeI[Kf] and KpnI and inserted into pUCDNA3 cut with EcoRI[Kf] and KpnI to give pCF358. The E1A-HD2 sequence was isolated from pALP8 with BsmBI

and HpaI and inserted into pCF358 to give pCF387. pCF358 was cut with SphI and cloned into pCF34 to make pCF389, the pre-gap repair vector containing Tcf-E1A, Tcf-E1B, and Tcf-E4 promoters. pCF387 was cut with KpnI and XhoI and inserted into pCF389 to give pCF393, the E1A-HD2 pre-gap repair vector. Finally, the Tcf-E1A/E1A-HD2/Tcf-E1B//Tcf-E4 cassette was isolated from pCF393 by PacI and inserted into pCF1 to produce the gap-repair vector pCF398. The vector was cut with SalI and gap repaired in yeast with the AB23'4 backbone as described previously[86] to give AHB23'4. Viral genomic DNA was converted into virus by transfection of PacI digested YAC/BAC DNA into cR1 cells. The viruses were then purified, expanded and characterised as described in chapter 2.3.4.

2.5. Tcf-viruses expressing yeast cytosine deaminase

2.5.1. Introduction

As stated in chapter 1.2.4, clinical trials have shown that oncolytic agents are not potent enough when used in monotherapies. To increase viral toxicity, viruses are made to express prodrug-activating enzymes, which convert a prodrug into its toxic form only in cells that express the transgene. The advantage of this approach is that the toxic effect is limited to the period of exposure to the prodrug, which itself is harmless.

The enzymes most commonly used for prodrug activation are HSV thymidine kinase (tk) and *E.coli* or *S.cerevisiae* cytosine deaminase (CD), although many other prodrug activating enzymes are being studied.[97,136] Cytosine deaminases convert the prodrug 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU). 5-FU is a widely used cytotoxic drug which is metabolised to fluorinated ribo- and deoxyribonucleotides which have multiple effects, including inhibition of thymidylate synthase, altered DNA stability, induction of single- and double-strand breaks, and decreased RNA stability.

In devising a strategy for expression of a suicide gene, one can choose between use of an early and a late transcription unit. Early expression risks killing the virus if the drug interferes with viral DNA replication.[79,203] Late expression of suicide genes is more attractive because replication can increase the number of transcription templates to many thousands of copies. Provided viral replication is restricted to tumour cells, genes expressed from late promoters should also be restricted to tumour cells. Therefore, there is no *a priori* reason to use a tumour specific promoter. Expression of a late gene is possible by making a fusion protein, by splicing a new exon into an existing transcript[112] or by reinitiation of translation from an internal ribosome entry site (IRES).[83,213]

To test whether late expression of a suicide gene could increase the toxicity of our Tcf viruses, I decided to construct two viruses that express the yeast cytosine deaminase gene (yCD) late during the viral cycle of the A4 virus. Because the early phase of the life cycle was already controlled by

the Wnt pathway, I could use an endogenous late promoter. For late expression in Ad5 there was a choice between using the major late promoter and the E2 late promoter. The E2 genes are also expressed from the E2 early promoter, making it unlikely that a linked therapeutic gene would only be expressed with late kinetics. The major late promoter drives expression of five groups of transcripts (L1 to L5) with common 5' exons (encoding the tripartite leader) and differing polyadenylation (polyA) sites. The major late transcripts encode mainly viral core and capsid proteins. I chose to express yCD from the major late promoter using either an internal ribosome entry site (IRES) or by alternative splicing of a new exon analogous to the Ad41 long fibre exon.

Both strategies (IRES or alternative splicing) led to replication-dependent expression of the transgene. yCD was expressed in higher amounts from the IRES-yCD virus (AIC4) than from the splice-yCD virus (ASC4). Northern blotting and RT-PCR confirmed that yCD was present in a bicistronic fibre-IRES-yCD messenger in the AIC4 virus. In ASC4, yCD was spliced from the tripartite leader like any late transcript. Nevertheless, most of the yCD-containing transcripts also contained the fibre sequence upstream of the suicide gene, indicating that the yCD splice acceptor sequence and the fibre polyadenylation signal should be improved. Viral burst size was reduced by less than ~10-fold by 5-FC, showing that expression of yCD as a late gene is compatible with virus replication. Cytopathic effect assays in colon cancer cell lines showed that both yCD viruses have ~10-fold increased toxicity in the presence of the prodrug 5-fluorocytosine (5-FC), which is converted to 5-fluorouracil (5-FU) by yCD. The largest gain in toxicity was seen in HT29 colon cancer cells, which are the least permissive colon cancer cells for the parental virus, indicating that the new 5-FC/yCD viruses may have broader applications for colon cancer therapy than their predecessors.

2.5.2. Results

Virus constructs

Yeast cytosine deaminase was used because it has a lower Km and higher Vmax than the bacterial enzyme.[104,183] The yCD coding sequence was inserted at the end of the L5 transcript in A4.[82] Two viruses were produced (AIC4 and ASC4, Figure 2.25). The AIC4 virus uses the encephalomyocarditis virus (EMCV) IRES to convert the L5 transcript into a bicistronic mRNA. The ASC4 virus uses the splice acceptor sequence from the Ad41 long fibre gene to splice the yCD cassette onto the tripartite leader exons of the major late transcript. A polyA signal was placed between the fibre and yCD genes in the ASC4 virus, with the intention of splitting the existing L5 transcript into new L5 and L6 transcripts encoding fibre and yCD, respectively. The yCD insertion contributes 520 bp to ASC4 and 1071 bp to AIC4, yielding total genome sizes that are only slightly larger than normal (Figure 2.25).

Both viruses grow as well as wild type Ad5 on SW480 cells, which have high Tcf activity and were used as producer cells. The viruses have a particle/pfu ratio approximately 5-fold higher than the parental virus, an increase that could be explained by the increase in genome size or a slight delay in fibre expression, leading to fewer infectious particles.
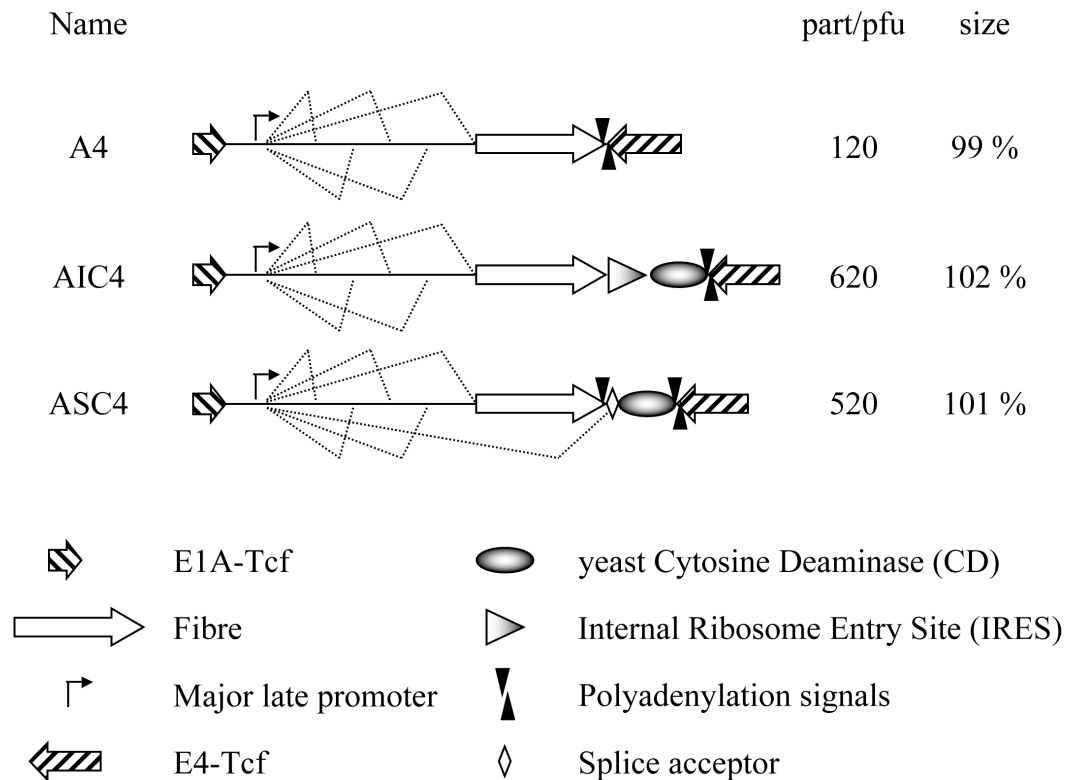
Figure 2.25: Adenoviruses used in this study. The name summarises the structure of the virus: A, 4 = Tcf sites in the E1A and E4 promoters; C = yCD; I = IRES; S = Ad41 splice acceptor. Size: the size of the viral genome relative to wild type Ad5. part/pfu: the ratio of particles measured by OD_{260} to plaque forming units measured on SW480 cells.

yCD is expressed with late kinetics

SW480 is a colon cancer cell line in which the A4 virus replicates slightly better than wild-type Ad5; Hct116 and HT29 are colon cancer cell lines with lower Tcf activity that are less permissive for A4 replication.[34,82] To check yCD expression from the new viruses, cell extracts were collected at various times after infection and western blots were probed for yCD and viral proteins. Despite the changes to the L5 transcript, fibre was expressed to similar levels by all three viruses, except for a slight delay and reduced level in SW480 with AIC4 (Figure 2.26). yCD expression was detectable in all three cell lines, with stronger expression from AIC4 than ASC4 (Figure 2.26). To determine whether yCD is expressed as a late gene, cells were treated with cytosine arabinoside (ara-C) to inhibit viral replication. This had no effect on expression of early genes (E1A and DBP) but blocked expression of yCD and fibre, showing that these behave as late genes.

Figure 2.26: Western blot for E1A, DBP, fibre and yCD at the indicated times after infection of colon cancer cell lines in the presence or absence of ara-C.

E2 and E4 expression in normal cells

Normal human small airway epithelial cells (SAECs) and lung fibroblasts (HLFs) were infected with the yCD viruses to test whether the A4 backbone retains its sp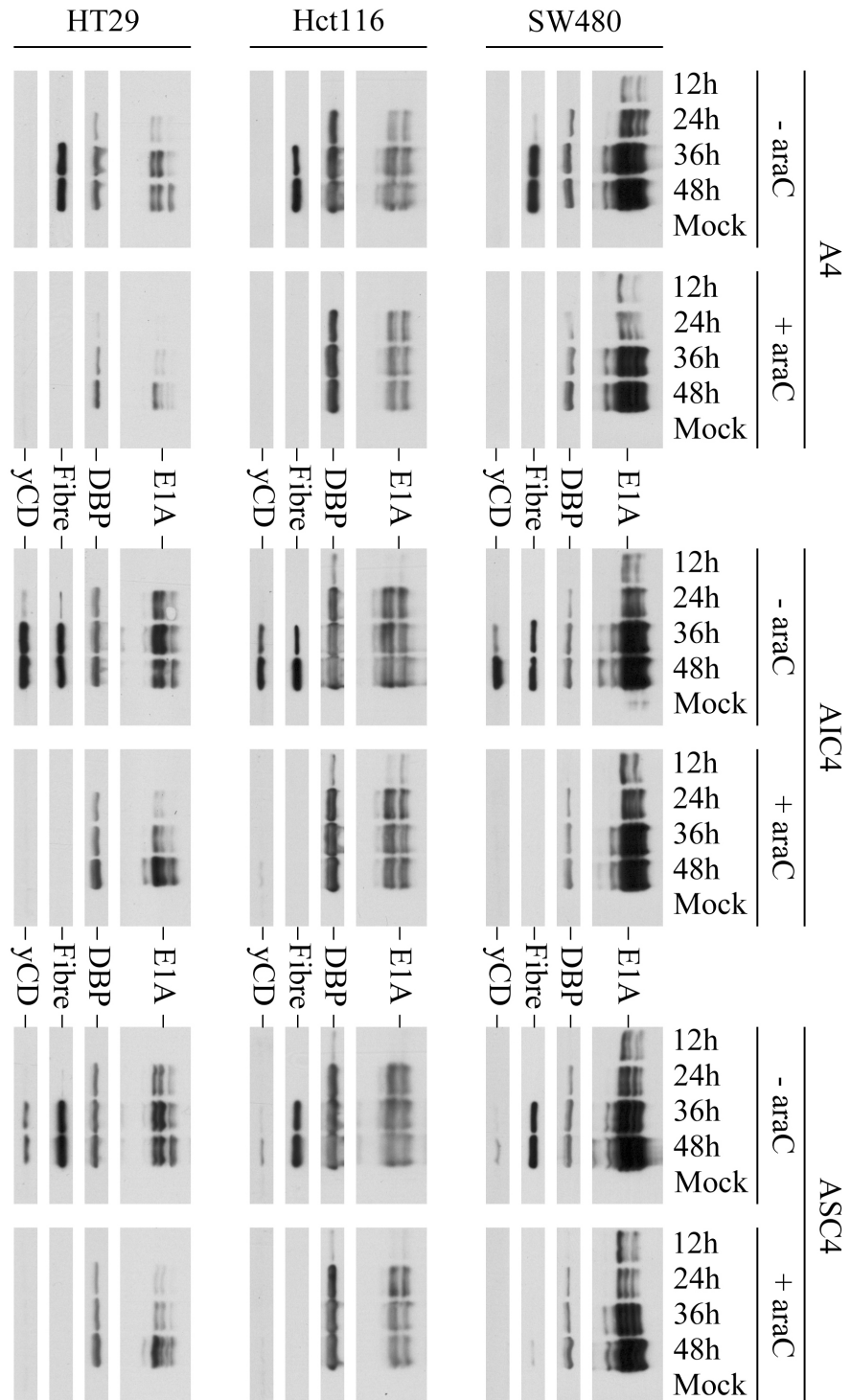ecificity for tumour cells after insertion of the transgene. Unexpectedly, both yCD viruses expressed DBP, and the ASC4 virus even expressed a small amount of fibre protein (Figure 2.27a). This could be caused by contamination of the viral batch with wild type virus, but this possibility was excluded by rigorous checking of the virus preparations using PCR primers specific for the wild type E1A promoter. DBP expression was not blocked by the inhibitor of DNA replication ara-C, showing that DBP was expressed from an early promoter. This could be explained by transactivation of an early promoter by an enhancer embedded in the yCD sequence or by a change in splicing of E2 transcripts initiated at an upstream promoter. To define the start site of the offending transcripts, rapid amplification of cDNA ends (RACE) was performed using RNA from SAECs harvested 48 hours after infection. The major RACE RT-PCR product had the same size in cells infected with wild type Ad5 as with the yCD viruses (data not shown). Sequencing of cloned RACE cDNAs showed that DBP was mainly expressed from the canonical E2 early promoter, ruling out abnormal splicing or transcription initiation at a cryptic promoter within the yCD sequence. The E2 early promoter is regulated by E1A and E4 orf6/7. Transactivation by E1A seems unlikely because E1A expression was not deregulated in normal cells infected with the yCD viruses, except for a small increase at late time points when the template copy number had probably increased (Figure 2.27a & b). An alternative possibility is that E4 orf6/7 expression is deregulated in the yCD viruses. This could be explained by transactivation of the E4 promoter by a fortuitous enhancer in the yCD gene, particularly given the proximity of the two sequences in the viral constructs. Western blotting for E4 proteins confirmed that E4 is indeed deregulated in the yCD viruses in SAECs and HLFs (Figure 2.27a & b).
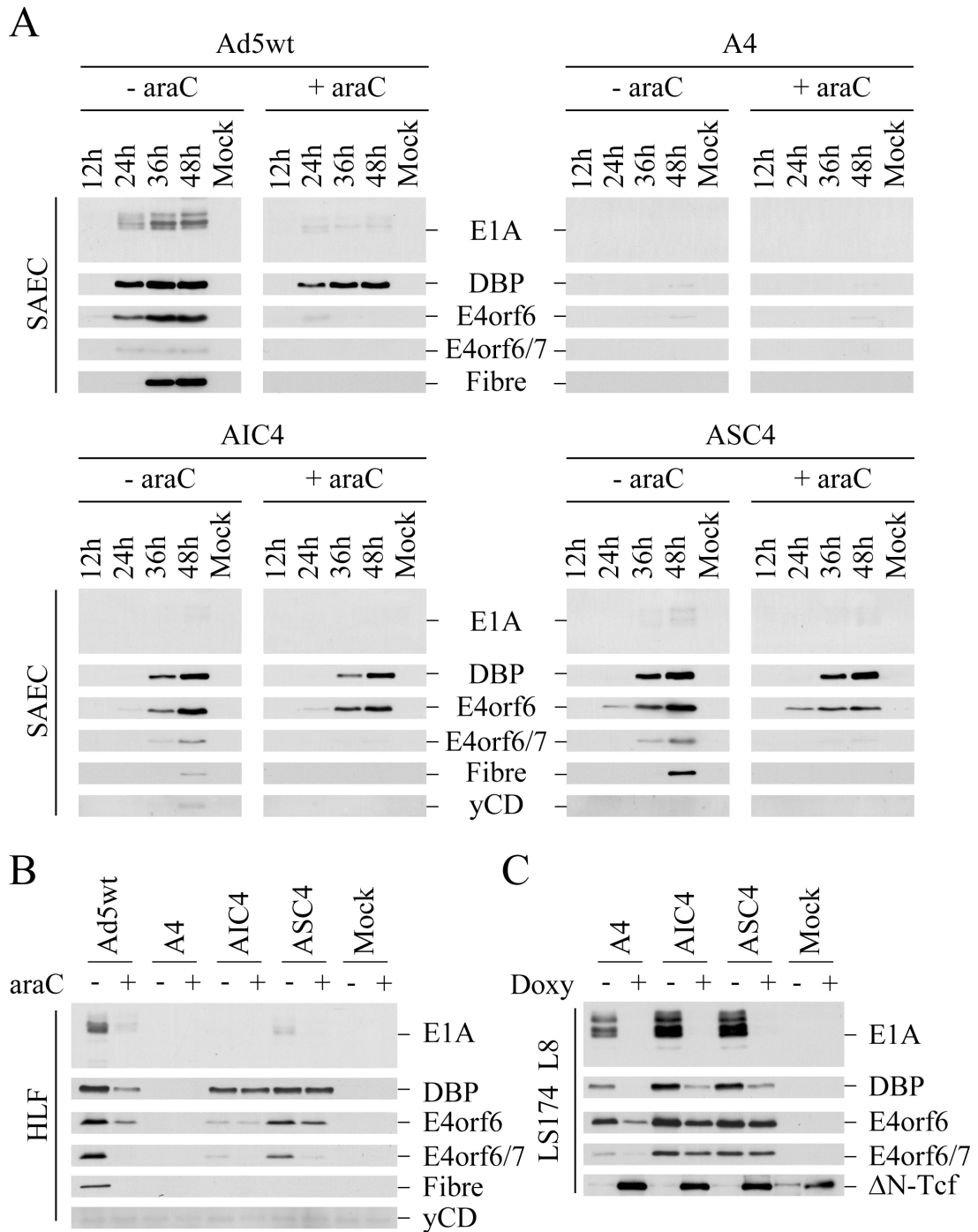
Figure 2.27: (A) and (B) Western blots for E1A, DBP, E4orf6, E4orf6/7, fibre and yCD at the indicated times after infection of SAECs (A) and 48 hours after infection of HLFs (B) in the presence or absence of ara-C. (C) Western blot for E1A, DBP, E4orf6, E4orf6/7 and Tcf4 12 hours after infection of LS174 L8 cells in the presence or absence of doxycycline.

To test the ability of the viruses to respond to activation of the Wnt signalling pathway, LS174T colon tumour cells with a dominant negative Tcf4 (DN-Tcf4) gene expressed from a tetracycline-inducible promoter (LS174 L8)[244] were infected with the Tcf regulated viruses (Figure 2.27c).

DN-Tcf4 abolished E1A expression, as expected given the presence of Tcf sites in the E1A promoter, but only partially inhibited DBP expression, showing that additional factors regulate DBP expression in these cells (Figure 2.27c, lanes plus doxycycline). One of these factors is probably E4 orf6/7, because E4 genes were expressed constitutively and failed to respond to DN-Tcf4 inhibition (Figure 2.27c).

Fibre expression was detectable at 48 hours in SAECs infected with the yCD viruses (Figure 2.27a). Despite higher fibre expression with ASC4, yCD was only detectable in SAECs infected with the AIC4 virus (Figure 2.27a), presumably because the IRES is more efficient than the Ad41 splice. yCD was not detectable by western blotting in HLFs at any time point, although this does not rule out weak expression below the detection limit of the antibody (Figure 2.27b). In summary, western blotting shows that the yCD viruses have lost some of their specificity for cells with Tcf activation, most likely because of transactivation of the E4 promoter by a fortuitous enhancer in the yCD sequence. To address this question, the yCD sequence was added downstream of the luciferase gene in a Tcf-E4 reporter plasmid, but failed to activate the Tcf-E4 promoter in the non-permissive cell line HeLa (data not shown). Although this simple assay might not reflect the context of a viral infection, it indicates that the yCD sequence itself is not able to directly transactivate nearby promoters. Alternatively, it is possible that the E4 promoter already has a background activity in Hela cells that prevents significant activation by the yCD sequence.

The exogenous splice acceptor is used correctly in the ASC4 virus

To determine whether the yCD cassette functions correctly as an L6 transcript in the ASC4 virus, the structure of the yCD mRNA was examined by northern blot analysis and RT-PCR. RNA was extracted from infected HT29 cells and northern blots were hybridised to fibre and yCD probes (Figure 2.28a). AIC4-infected cells gave a 3.0 kb band with both probes that had the size expected for the fibre-IRES-yCD mRNA. The ASC4-infected cells gave a 2.5 kb band with both probes that was bigger than the expected wild type fibre or yCD mRNAs (2.0 kb and 0.7 kb, respectively). To

ascertain the nature of this RNA, an RT-PCR was performed with primers in the tripartite leader and yCD. This confirmed the presence of the major 2.5 kb transcript observed on the northern blot (Figure 2.28b, ASC4 lane). The RT-PCR also showed 0.7 kb bands potentially corresponding to correctly spliced L6 RNA. The 2.5 and 0.7 kb PCR products from the ASC4-infected cells were cloned and sequenced. A schematic description of the observed transcripts is shown in Figure 2.28c.
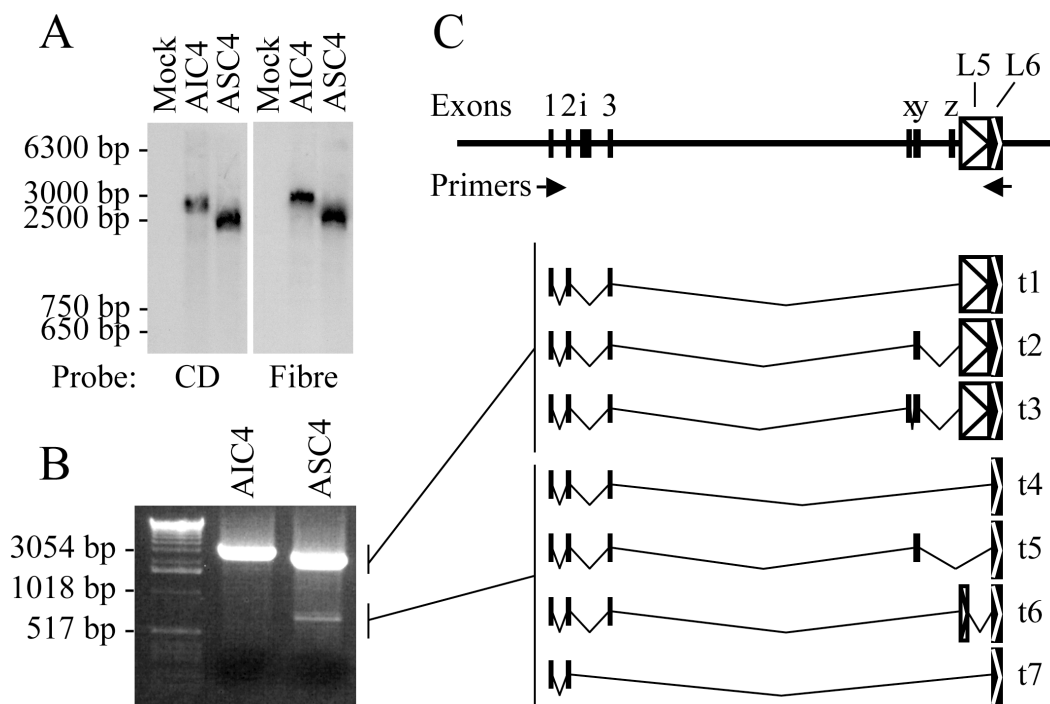
Figure 2.28: (A) Northern blots of RNA from HT29 cells 48 hours after infection with AIC4 or ASC4 viruses. The blots were probed for yCD or fibre. (B) RT-PCR of the same RNA using primers for the tripartite leader and yCD. (C) Schematic diagram showing the structure of the transcripts in (B). t1-7, transcripts.

The 2.5 kb band corresponds to yCD transcripts that contain the fibre gene preceded by the tripartite leader either alone (labelled t1 in Figure 2.28c) or combined with the x and y leaders (t2, t3). The presence of these transcripts is explained by failure of the prototypic L5 transcripts to use the polyA signal placed between the fibre and yCD genes. The lower bands correspond to mRNAs that use the exogenous Ad41 splice acceptor to create the desired new L6 transcript. The tripartite leader was correctly used, either alone (t4) or in conjunction with the y leader (t5). Two less

abundant transcripts were observed (t6 and t7), which nevertheless still used the Ad41 acceptor. I conclude that the Ad41 splice acceptor is functioning correctly but weakly and that the polyA signal between fibre and yCD is used inefficiently if at all. The smaller amount of correctly spliced yCD transcripts readily explains the lower yCD expression seen on western blots with the ASC4 than the AIC4 virus (Figure 2.26).

Viral burst size in the presence of 5-FC

To test whether 5-FC interferes with virus replication, I performed burst assays on colon cancer cell lines (Figure 2.29a). The cell pellet and culture supernatant were tested separately to detect any effect on virus release. Viral burst size was higher in SW480 than Hct116 or HT29 cells with all three viruses, as expected. In the absence of 5-FC, the yCD viruses replicated at least as well as A4, despite their higher particle to pfu ratio. Less virus was detected in the pellet fraction in Hct116 and HT29 cells after 5-FC treatment. The amount of virus in the supernatant was little changed by 5-FC treatment, indicating that the drug had little effect on virus release. Thus, treatment with 5-FC has a small effect on the burst size of the AIC4 and ASC4 viruses.

To test whether the increased E2 and E4 expression from the yCD viruses in normal cells decreases the selectivity of the viruses at the level of viral replication, burst assays were performed in normal cells. In SAECs, the A4 virus was more than 1000-fold attenuated relative to wild type Ad5 (Figure 2.29b). The yCD viruses replicated 10 to 100-fold better than A4 in these cells. 5-FC had a small effect on replication comparable to that in colon cancer cell lines, consistent with the yCD expression seen by western blotting in SAECs (Figure 2.27a). In HLFs, the A4 virus was ~1000-fold attenuated compared to wild type Ad5 (Figure 2.29c). The yCD viruses were again less selective than A4, but still showed ~100-fold attenuation relative to wild type Ad5. In summary, the yCD viruses replicate better than the A4 virus in non-permissive cell lines, but remain attenuated compared to wild type Ad5.
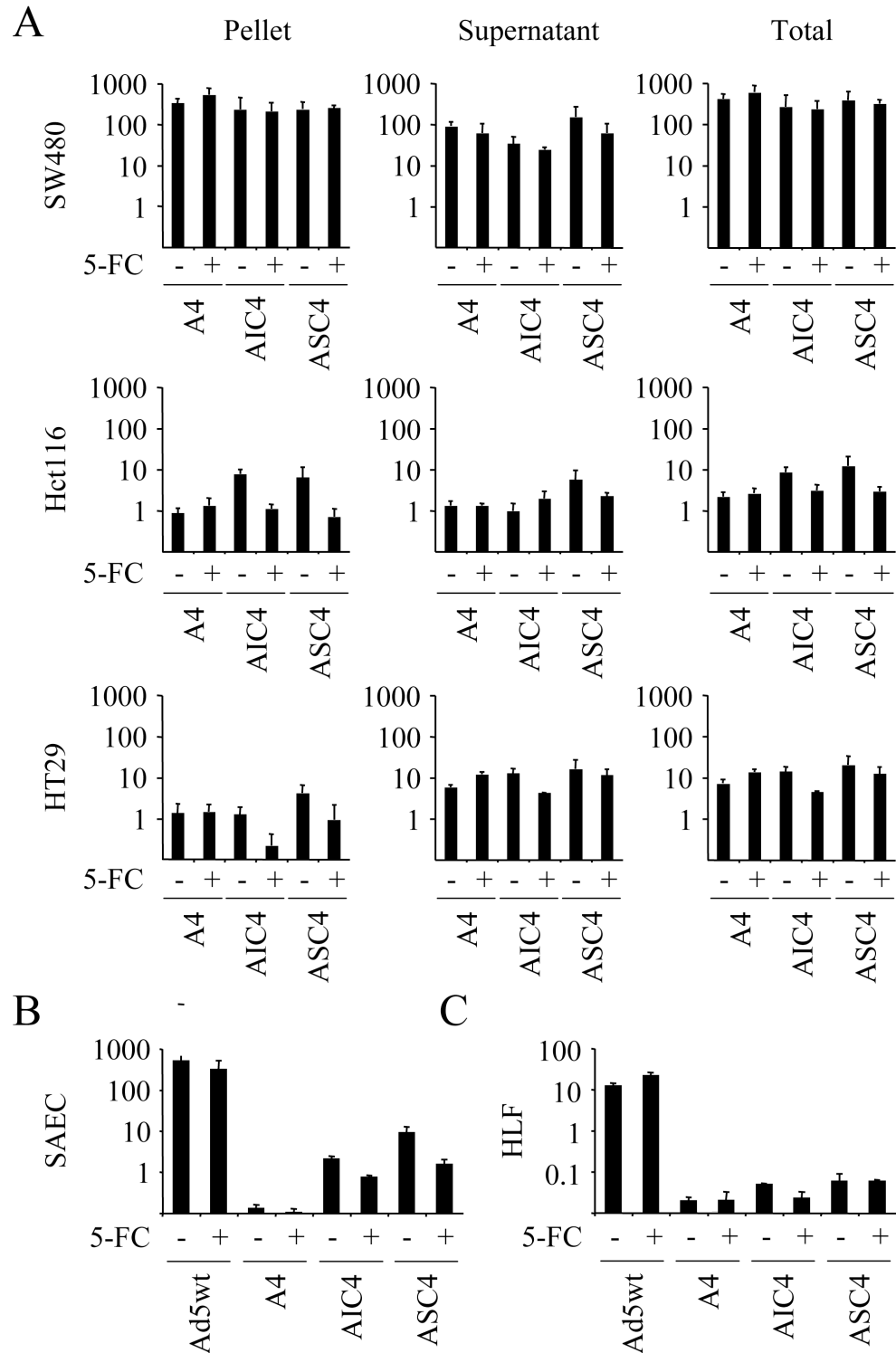
Figure 2.29: Viral burst assays in the presence or absence of 800 μM 5-FC. (A) Colon cancer cell lines, (B) SAECs, and (C) HLFs were infected with 1 pfu/cell and collected 48 hours post-infection. The titres of virus in the cell pellet and the supernatant (A) or both combined (B and C) were measured by plaque assay. Values are expressed as pfu produced per pfu used for infection.

5-FC increases the toxicity of the yCD viruses

Before testing the toxicity of the yCD viruses, I first looked at the sensitivity of different cell lines to 5-fluorouracil to test their responsiveness to the drug (Figure 2.30a). Cells were grown in the presence of various 5-FU concentrations for 4 days and stained with crystal violet, to mimic the readout of a cytopathic effect (CPE) assay. SW480 cells were at least 10-fold more resistant to 5-FU than the other cell lines. Hct116 cells with a homozygous deletion of the tumour suppressor gene p53 show a greatly reduced apoptotic response to 5-FU but were only slightly more resistant than the parental cells in this assay.[35] The cell lines were then infected with 10-fold dilutions of virus in the presence or absence of 800 μM 5-FC (Figure 2.30b to d, and "E" in e). In every cell line tested, 5-FC had no effect on the toxicity of the parental A4 virus but increased the toxicity of the yCD viruses 5 to 10-fold (compare lanes + and -). The gain in toxicity was comparable between AIC4 and ASC4, despite evidence from western blotting that AIC4 gives higher yCD expression than ASC4 (Figure 2.26). This could indicate that low levels of enzyme are sufficient for production of toxic amounts of 5-FU, or it may simply reflect the longer duration of the CPE assay. Deletion of p53 in Hct116 did not confer resistance to 5-FC. The biggest effect was seen in HT29, the colon cancer cell line least sensitive to the parental virus, probably because they express the most yCD.

Inspection of the cultures revealed that 5-FC increased the toxicity of the yCD viruses as early as two days after infection. To test whether the toxicity would increase by application of the drug after completion of the first cycles of viral replication, I compared addition of 5-FC either directly after infection (Figure 2.30e, "E") or four days later (Figure 2.30e, "L"). Late administered 5-FC was not toxic, except for a small effect with the AIC4 virus, which expresses the highest amount of yCD. Earlier addition of 5-FC exposed the cells to the drug for twice as long, which may account for the increased toxicity. The ability of the virus to kill the cells at a multiplicity of infection (m.o.i.) below 1 pfu/cell could be explained either by viral replication and spread in the presence of the drug or by a bystander effect caused by release of 5-FU from infected cells.
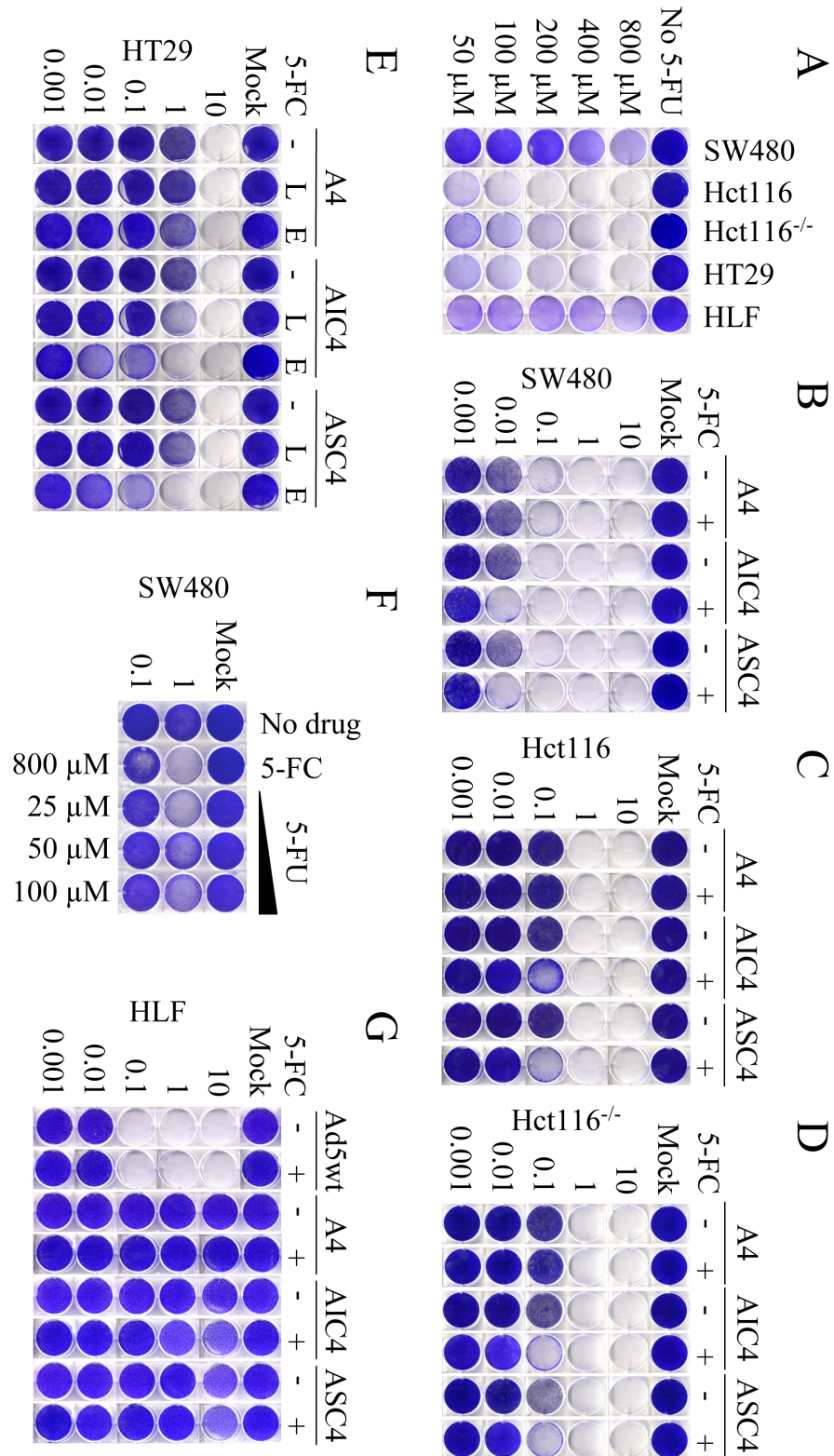
Figure 2.30: (A) Sensitivity of colon cancer cell lines and HLFs to 5-FU. Cells were stained four days after addition of the drug. (B to E, G) Cytopathic effect assays using 10-fold dilutions of virus. 800 µM 5-FC was added immediately after infection, except for HT29 (L, late) where it was added four days after infection. Cells were stained 5 (SW480), 7 (Hct116 and Hct116$^{-/-}$), or 8 (HT29 and HLF) days post-infection. (F) SW480 were infected with AIC4 at an m.o.i. of 1 or 0.1 and 800 µM 5-FC or 25-100 µM 5-FU was added either immediately after infection. Cells were stained 4 days after infection.

If the increase in CPE is caused by conversion of 5-FC to 5-FU, it should be possible to mimic the effect by treating infected cells with 5-FU instead of 5-FC. This can only be tested with low doses of 5-FU, because higher doses inhibit cell growth even in the absence of virus. SW480, the cells most resistant to 5-FU, were used to compare the effects of 5-FC and 5-FU after infection with the AIC4 virus (Figure 2.30f). The assay was stopped after 4 days, compared to 8 days in Figure 2.30b, which explains why a higher m.o.i. was required to kill the cells. At 25 μM 5-FU there was an increase in CPE in the cells infected at an m.o.i. of 1. The gain from combining the two treatments was less clear at higher 5-FU concentrations, probably because of combined inhibition of virus and cell growth by the drug. The advantage of 5-FC was apparent from the fact that it could be given at a much higher concentration without harming the uninfected cells (Figure 2.30f, mock), yet increased the CPE of virus at an m.o.i. of 0.1 whereas 5-FU required an m.o.i. of 1 (Figure 2.30f).

Finally, the toxicity of the viruses was tested in normal cells (HLFs, Figure 2.30g). All three Tcf viruses were ~1000-fold less toxic than wild-type adenovirus type 5. AIC4 and ASC4 started to show some CPE at an m.o.i. of 10. This correlates with the expression of DBP and fibre seen in Figure 2.27. 5-FC had a marginal effect with AIC4, presumably due to yCD expression below the detection limit of western blotting.

In conclusion, 5-FC increased the toxicity of the yCD viruses in all colon cancer cell lines tested but had only a minor effect in normal fibroblasts. Despite the difference in the efficiency of yCD expression from the AIC4 and ASC4 viruses, there were only small differences in the toxicity of the two viruses. The biggest gain in therapeutic response was seen in HT29 cells, which show the least response to the parental virus alone.

2.5.3. Discussion

I used reinitiation of translation or alternative splicing to express the yCD transgene from the major late promoter in the A4 backbone. The IRES-yCD and splice-yCD cassettes raised the size of the genome to 102% and 101% of wild type size, respectively. This is below the published 105% packaging limit.[18] I preferred addition to replacement because replacement of a viral gene carries the risk that the virus might be less active in vivo. For instance, the E3 genes are non-essential and can be replaced with therapeutic genes,[14,111-113] but retention of the E3 region has been shown to increase the efficacy of oncolytic adenoviruses in mouse xenograft models.[230,263] Similarly, ADP is important for cell lysis and is not an ideal candidate for replacement in an oncolytic virus, although delayed lysis can permit more prolonged transgene expression.[112] The E3B region is deleted in many laboratory strains of Ad5, including the dl1520 E1B-deleted virus used by the Onyx group in clinical trials, but deletion of the E3B 14.7 kD gene increases the neutrophil response and could hasten clearance of the virus *in vivo*.[91,248] Recently, deletion of E3gp19k has been found to increase viral spread in an immunocompetent mouse model, although the basis of this effect is not clear.[248]

The L5/E4 junction was chosen as the site for insertion of yCD because use of distal splice sites in the major late transcript is blocked early in infection. Use of a putative L6 transcript would thus guarantee the maximum restriction of expression to cells that are committed to viral replication, both at the level of promoter activation and splice site selection. This is desirable for a suicide gene whose expression is not restricted to tumour cells by use of a tumour-specific promoter. yCD expression was blocked by treatment of cells with ara-C, which prevents viral DNA replication, demonstrating that yCD is expressed with late kinetics.

Of the two methods tested for expressing yCD, the IRES gave higher expression. Expression of a transgene using an IRES in this site was recently demonstrated with p53.[213] One disadvantage of the EMCV IRES is its relatively large size (588 bp). A recent study found that the eIF4G IRES, which is only 339 bp long, gave substantially higher expression than the

EMCV IRES.[256] If the major concern is to avoid increasing the size of the virus while maintaining a full complement of viral genes, the most attractive ways to express a suicide gene are fusion to a viral protein or alternative splicing.

Fusion to a late gene in the E3 region is an interesting possibility; fusion to a structural gene might result in loss of specificity if active enzyme could be packaged and escape from the endosome immediately after infection. At first glance, the complexity of splicing of the major late transcript makes alternative splicing a less attractive option. In fact, the enteric adenoviruses Ad40 and Ad41 prove that it is possible to insert an additional L6 splicing unit in the major late transcript.

As a first step to test the feasibility of adding an L6 transcript to Ad5, I inserted the Ad41 L6 splice acceptor site between the fibre and yCD genes. Sequencing of mRNAs cloned from infected cells showed that the Ad41 splice acceptor was used correctly, but RT-PCR and northern blotting both indicated that it was only used by a minor group of transcripts. Furthermore, the L5 transcript did not use its new polyA signal but instead continued to the putative L6 polyA signal. These observations demonstrate the feasibility of adding an L6 transcript to Ad5 after transfer of only a minimal stretch of Ad41 sequence. Further development could include transfer of a larger piece of the Ad41 DNA separating the long and short fibre genes to improve the efficiency of splicing and termination, or inclusion of binding sites for virus-dependent splicing enhancer factors such as the 3VDE sequence to reinforce the restriction of expression to the late phase of infection.[172]

Despite the low level of correct splicing, western blotting showed that yCD was indeed expressed from the ASC4 virus. The simplest interpretation is that it was translated from the correctly spliced "L6" transcripts, although translation from the more abundant "L5" transcripts by reinitiation cannot be excluded. The response to 5-FC of the AIC4 and ASC4 viruses was almost identical in the colon cancer cell lines, and there was an effect on replication and CPE in normal cells despite low or undetectable yCD expression in these cells by western blotting. This probably indicates that the level of yCD required to convert useful amounts

of 5-FC to 5-FU is low and well within the range that can be achieved with the virus. Since the therapeutic gain is only seen in the presence of the drug, the fate of 5-FC in infected cells is of great interest. At the dose used, 100% conversion would be required to kill SW480 cells unless the 5-FU were concentrated in the cells or more efficiently used than when supplied in the medium. A possible explanation is that the virus and prodrug act synergistically. Disentangling this issue with selectively replicating viruses is not simple, because the virus itself invariably shows some toxicity during the late phase of infection.

The particle to pfu ratio of the yCD viruses was five-fold higher than that of the parental virus. The difference was not associated with an obvious small plaque phenotype or a reduction in CPE or burst size. It could be due to inefficient packaging of the over-sized genome, but the increase in genome size was small, particularly for ASC4, and certainly within the published 105% limit.[18] An alternative explanation is that reduced fibre expression may have resulted in the production of some fibreless viruses. Deletion of the E4 region has previously been noted to have this effect.[32] The reduction in fibre expression was most obvious at early time points, after which the yCD viruses showed normal fibre expression.

One unexpected finding was that insertion of yCD increased DBP expression in normal cells. This expression was not blocked by ara-C, suggesting that it came from an early promoter. In addition to transcripts originating from the well characterised E2 early and late promoters, the classic Ad2 transcript map shows E2 transcripts originating from the E3/L5 junction and the E4 promoter.[31] I can rule out increased expression of DBP from the latter sites, or from a novel promoter in the yCD gene itself, as transcript mapping by RACE showed that the offending transcripts began mainly at the prototypic E2 early promoter. Direct transactivation of this promoter by an enhancer in the yCD sequence is possible. Indirect transactivation by E4 orf6/7 is also possible, as I have shown that E4 expression is deregulated in these viruses. Nevertheless, one cannot favour either possibility based on the available data. Reporter assays indicated that the yCD sequence was not able to transactivate a nearby promoter (data not shown), but this might not reflect the viral situation because the reporter

construct is quite different from the viral genome and transfection experiments are not directly comparable to viral infections. The yCD viruses had a particle/pfu higher than the A4 virus and all the m.o.i were expressed as pfu/cell. Thus, it is formally possible that a higher amount of defective viral particles resulted in non-specific transactivation of the early promoters. In that respect, proteins of the capsid like pIX have been reported to activate transcription from TATA-containing promoters.[163]

E2 expression in normal cells leads to virus replication and yCD expression, which can be seen as a slight decrease in the confluence of 5-FC-treated HLFs infected with the highest dose of virus and a reduction in the burst size of 5-FC-treated SAECs. It is worth pointing out in this context that the Tcf virus backbone used for these experiments has the least selectivity of our family of Tcf viruses. If greater selectivity is required, I have ample scope for increasing it by addition of Tcf sites to other early promoters.[82] Alternatively, it might be possible to map the putative enhancer in the yCD sequence and then destroy it by mutagenesis if such an element is active in the context of the virus.

I have shown that 5-FC treatment of colon cancer cells infected with an oncolytic virus expressing yCD from the major late promoter increases the cytopathic effect of the virus by about 10-fold. The magnitude of the improvement appears small because Tcf viruses are already highly active in these cells. In SW480, for example, the parental virus can kill the cells at an moi of 0.01. The largest gain in activity was seen in HT29, which are relatively resistant to Tcf viruses because of low Tcf activity. The gain in activity can be unequivocally attributed to the action of yCD on 5-FC, because both are required to see the effect. It is a complex phenomenon resulting from the combination of multiple competing factors. Minimally, these include the efficiency of conversion of 5-FC to 5-FU, the sensitivity of viral and cellular replication to 5-FU, the toxicity of 5-FU and perhaps bystander effects.

Adenoviral replication has been reported to improve at 4 μM 5-FU and decline at 250 μM 5-FU.[17] The results of that study may not exactly translate to this work because of important differences in virus design, but

the overall conclusion was that drugs blocking cells in the S or G2 phases of the cell cycle generally potentiate virus replication. In particular, camptothecin and cisplatin were both compatible with virus replication, even if topoisomerase I is required for in vitro viral DNA replication and DNA inter-strand cross-links should stall the viral polymerase. This is encouraging because the two chemotherapeutics commonly given with 5-FU for colon cancer are irinotecan, a topoisomerase I inhibitor related to camptothecin, and oxaliplatin, a cross-linking agent related to cisplatin. Combination therapy with virus and 5-FU or cisplatin was previously found by the Onyx group to give better results than virus alone in xenografts[121] and gave encouraging results in patients.[135,199] The use of non-toxic prodrugs such as 5-FC would represent a considerable gain for patients. The efficacy of 5-FC treatment can be further increased by expressing enzymes that hasten the conversion of 5-FU to 5-FdUMP, such as uracil phosphoribosyl transferase or thymidine phosphorylase.[52,53] Of particular relevance to colon cancer, it is possible to reduce the dose of irinotecan by expressing carboxyesterase, which converts irinotecan to SN38, the active metabolite.[253] Yet another option for colon cancer is to express nitroreductase, which converts CB1954 to a bifunctional alkylating agent.[155]

Preliminary xenograft experiments showed that Tcf viruses are able to slow tumour growth when administered intravenously and indicated that 5-FC further increases the toxicity of AIC4 *in vivo* (K. Homicsko, pers. comm.). An improved protocol will certainly lead to more significant differences. 5-FC treatment alone of xenografts injected with a replicating E1B-deficient adenovirus expressing a bacterial CD gene fused to HSV tk (Ad5-CD/TK*rep*) was unable to eradicate tumours.[203] To achieve cures with that virus, it was necessary to give two prodrugs (5-FC and ganciclovir) combined with radiotherapy. Following infection with Ad5-CD/TK*rep*, expression of the CD-tk fusion protein starts early but only reaches high levels following amplification of the template copy number by viral replication. Ganciclovir was considerably more effective at blocking viral replication than 5-FC, although infection was severely attenuated by 5-FC at the concentration used in our study.[79,203] Monotherapy for cancer is rare, and most groups now expect to see viral therapy used only in combination

with other treatments. Besides major differences in the way viral replication is targeted to tumours and the wide range of different prodrug/enzyme systems under development, additional layers of complexity are provided by the need to improve tumour targeting at the level of the cell surface receptor and the need to evade neutralising antibody after intravascular injection.[15,114,123,143,194,200]

In summary, I have developed oncolytic adenoviruses that express a prodrug activating enzyme with late kinetics. They are selective for cells with activated Wnt signalling, but less so than the parental A4 virus. This might be due to inappropriate transactivation of viral early promoters by an enhancer in the yCD sequence or simply reflect a higher particles/pfu ratio of the yCD viruses. Combination treatment of colon cancer cell lines with virus and prodrug gives a 10-fold increase in toxicity in vitro over that of virus alone. Moreover, preliminary *in vivo* experiments showed enhanced cytopathic effect in the presence of 5-FC. Taken together, Tcf-viruses expressing yCD late during infection are potential vectors for the treatment of colon cancer.

2.5.4. Materials and methods

Adenovirus mutagenesis

The fibre region (nucleotides nt 30470 to 33598) of adenovirus 5 (ATCC VR5) was cut with KpnI/XbaI and cloned into pUC19 to give pCF159. A SpeI site was inserted after the polyA site of the fibre by inverse PCR with primers AGTTTCTTTATTCTTGGGCAATGT (oCF67) and AGTCGTTTGTGTTATGTTTCAAC (oCF68) to give pCF277. yCD was cloned from S.cerevisiae genomic DNA by PCR with primers TCGCTAGCCAGGCACAATCTTCGCATTTCTTTTTTTCCAGATGGT GACAGGGGGAATGGC (oCF31) and TGACTAGTTATTCACCAATAT CTTCAAA (oCF32). The product was cut with NheI and SpeI (underlined) and inserted into the XbaI site of pCDNA3 (Invitrogen, Carlsbad, USA) to give pCF232.

The EMCV internal ribosome entry site (IRES) was cloned by PCR from the pSE280-IRES plasmid (gift of O. Zillian, ISREC). This plasmid contains the EMCV IRES of pCITE-1 (Novagen, Madison, USA) cut with EcoRI and BalI and cloned into the EcoRI/SmaI sites of pSE280 (Invitrogen, Carlsbad, USA). The IRES was amplified with primers ATGCTAGCGAATTCCGCCCCTCTC (oCF69) and ATACTAGTTATGC ATATTATCATCGTGTTT (oCF70), cut with NheI and SpeI (underlined) and inserted into the SpeI engineered immediately downstream of the fibre to give pCF274. This plasmid contains the full-length wild-type fibre followed by the EMCV IRES. The BfrBI site at the end of the IRES (bold) can be used to introduce a foreign gene, whose first codon is the ATG of the BfrBI site. The polyA site of fibre is embedded at the end of the coding sequence and was mutated by silent mutations (GAA TAA A to GAG TAG A, where the coding sequence remains Glu-Stop). To do so, the 5'-end of the fibre gene was amplified by PCR from pCF274 using primers GGAATTCGCTAGTTTCTCTACTCTTGGGCAATGTA (oCF77, contains the mutant polyA signal, underlined) and GGTGGTGGAGATGCTAAACT CACTTTGGTC (oKH9) and re-introduced into pCF274 using EcoRI and BstXI. The vector obtained after backcloning is pCF328. It contains the full-length wild-type fibre sequence with a mutant polyA site followed by the EMCV IRES. This viral sequence is in a pRS406 backbone.[220]

yCD was cloned by PCR with primers GTGACAGGGGGAATGGCA AG (oCF71) and TGACTAGTTTATTCACCAATATCTTCAAA (oCF76), cut with SpeI and inserted into the BfrBI/SpeI sites of pCF278, a K7-fibre but otherwise identical derivative of pCF274, to give pCF308. An extra A (bold) was added at the end of yCD (last two codons underlined) to create a polyA signal. The junction between the IRES and yCD was corrected by PCR to give pCF317. The IRES-yCD cassette of pCF317 was backcloned with AvrII and SpeI into pCF328 to obtain pCF330, the corresponding shuttle vector.

The splice acceptor sequence was synthesised in oCF31 and used with oCF76 to amplify yCD by PCR. The product was cut with NheI and SpeI and cloned into the SpeI site of pCF277 to give pCF298. The splice cassette

of pCF298 was backcloned with EcoRI into pCF260 to obtain pCF325, the corresponding yeast integrating vector.

The IRES-yCD (pCF330) or splice-yCD (pCF317) sequences were introduced into the vCF11 (A4) YAC/BAC[82] by two-step gene replacement in yeast[86] to obtain vpCF12 and vpCF13, respectively. Plasmids were checked by automated fluorescent sequencing on a Licor 4200L sequencer in the fibre region using primers IF272 (Fibre sense: GCCATTAATGCAG GAGATG) and IR281 (E4 antisense: GGAGAAAGGACTGTGTACTC).

Viral genomic DNA was converted into virus by transfection of PacI digested YAC/BAC DNA into cR1 cells. The viruses were then plaque purified on SW480 cells, expanded on SW480, purified by CsCl banding, buffer exchanged using NAP25 columns into 1 M NaCl, 100 mM Tris-HCl pH 8.0, 10% glycerol and stored frozen at -70°C. The identity of each batch was checked by restriction digestion. Particle counts were based on the OD_{260} of virus in 0.1% SDS using the formula 1 OD_{260} = 10^{12} particles/ml. Pfu titres were measured on SW480.[34] The clone names for AIC4 and ASC4 are vCF125 and vCF132, respectively.

Cell lines

SW480 (ATCC CCL-228), HCT116 (CCL-247) and HT29 (HTB-38) were supplied by ATCC. Human embryonic lung fibroblasts (HLFs) were supplied by Dr M Nabholz. $p53^{-/-}$ HCT116 were supplied by Dr B Vogelstein.[35] cR1 cells are C7 cells expressing myc-tagged ΔN-β-catenin (see chapter 2.3.4). LS174 L8 were supplied by Dr M van de Wetering[244] and grown in RPMI medium with 5% foetal calf serum (Invitrogen, Carlsbad, USA). All other cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% foetal calf serum. Clonetics small airway epithelial cells (SAECs) were grown in SAGM medium (Cambrex, East Rutherford, USA).

Western blotting

Cells were infected with 10 plaque forming units (pfu) per cell in DMEM. Two hours after infection, the medium was replaced with complete medium plus or minus 20 μg/ml of cytosine arabinoside (Sigma, St. Louis, USA). Cells were harvested at various times in SDS-PAGE sample buffer. E1A, DBP, Fibre and yCD were detected with the M73 (Santa Cruz Biotechnology, Santa Cruz, USA), B6,[196] 4D2 (Research Diagnostics Inc, Flanders, USA) and 2485-4906 (Biogenesis, Poole, England) antibodies, respectively. E4orf6 and E4orf6/7 were detected with the RSA3 antibody.[167] For LS174 L8 cells, dominant-negative Tcf expression was induced with 1 μg/ml of doxycycline (Sigma, St. Louis, USA). Tcf4 was detected with the N-20 antibody (Santa Cruz Biotechnology, Santa Cruz, USA).

Cytopathic effect assay

Cells in six-well plates were infected with ten-fold dilutions of virus in DMEM. Two hours after infection, the medium was replaced with complete medium containing or not 800 μM 5-fluorocytosine (Sigma, St. Louis, USA). Four days after infection, new medium was added. Late addition of 5-FC was performed at that time. After five to eight days (see legend to Figure 2.30), the cells were fixed with 4% formaldehyde in PBS and stained with crystal violet. For the sensitivity to 5-fluorouracil (Sigma, St. Louis, USA), the drug was added at various concentrations for four days before staining with crystal violet.

Virus replication assay

Cells in six-well plates were infected with 1 pfu per cell in DMEM. Two hours after infection, the medium was replaced with complete medium containing or not 800 μM 5-fluorocytosine (5-FC). 48 hours later, the medium and the cells were collected and centrifuged at 3000 rpm in a table-top centrifuge. The supernatant was collected, while the pellet was resuspended in medium containing 10% glycerol and lysed by three cycles of freeze-thawing. Cell extracts were obtained after centrifugation of the

cellular debris. Both supernatant and cell extract were tested for virus production by counting plaques formed on SW480 cells after 10 days under 0.9% Bacto agar in DMEM 10% FCS. Two independent infections were tested in triplicate for the cell extracts. One infection was tested in duplicate for the supernatant. For SAECs and HLFs, supernatant and cell extract were collected together and tested in duplicate.

Northern blotting and RT-PCR

HT29 cells were infected with 10 pfu per cell in DMEM. RNA was extracted with the Qiagen Rneasy midi kit following the manufacturer's instructions (Qiagen, Hilden, Germany). 10 μg total RNA per lane was resolved on a 1.2% agarose/1x MOPS/6.3% formaldehyde gel. RNA was transferred by capillarity with 20x SSC on positively charged membrane (Appligene, Strasbourg, France) and UV cross-linked to the membrane in a Stratalinker (Stratagene, La Jolla, USA). Northern blots were hybridised with random-primed ^{32}P-labeled probes corresponding to full-length cytosine deaminase (482 bp, PCR with oCF71 and oCF76) or a 468bp fragment of fibre (NheI to HindIII). The membranes were prehybridised in Church Buffer (0.5M NaPO$_4$, 1mM EDTA, 7% SDS, 1% BSA) for 2 hours at 65°C and hybridised in the same conditions overnight. Blots were washed in 2xSSC, 0.1% SDS at 65°C, and then in 1xSSC, 0.1% SDS at 65°C.

RT was performed with oligo-dT$_{12-18}$ (Amersham Biosciences, Little Chalfont, UK) and Superscript II reverse transcriptase (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. yCD was amplified with Pfu turbo (Stratagene, La Jolla, USA) using primers oCF76 and AGGATCCACTCTCTTCCGCATCGCTGTC (TPLupper). Bands were purified from a TAE agarose gel and 3' A-overhangs were added with Taq DNA Polymerase (Sigma, St. Louis, USA). The PCR product was cloned by TOPO TA cloning into pCR2-1-TOPO following the manufacturer's instructions (Invitrogen, Carlsbad, USA) and sequenced using primers AGGGTTTTCCCAGTCACGACGTT (M13fwd) and AGCGGATAACAA TTTCACACAGGA (M13rev).

RACE cloning of the DBP mRNA cap site

SAECs were infected with 10 pfu per cell in DMEM. RNA was extracted with the Qiagen Rneasy midi kit following the manufacturer's instructions (Qiagen, Hilden, Germany). Reverse transcription was performed with a DBP-specific primer (TTGTGATGAGTCTTCCT, oCF109) and Superscript II reverse transcriptase (Invitrogen, Carlsbad, USA). RNA was digested with RNase H (Invitrogen, Carlsbad, USA) after the reverse transcription. cDNA was purified on a Qiagen PCR purification column (Qiagen, Hilden, Germany) and polyadenylated with terminal deoxynucleotidyl transferase (Amersham Biosciences, Little Chalfont, UK) according to the manufacturer's instructions. The polyadenylated cDNA was then amplified by PCR using primers oCF110 (CTGCTCCTCTTCCCGACT) and T7-Oligo(dT). (GGCCAGTGAATTGT AATACGACTCACTATAGGGAGGCGGTTTTTTTTTTTTTTTTTTTT TTTVN, where V is G, A or C, and N is any nucleotide) with Taq DNA polymerase (Sigma, St. Louis, USA). The PCR product was cloned by TOPO TA cloning and sequenced with M13fwd and M13rev primers.

3. Perspectives

I have constructed a panel of Wnt-targeting replicating adenoviruses and shown that these viruses are selective for the Wnt pathway. These viruses are active in most of the colon cancer cell lines studied. A subset of CRC cells that have activated Wnt pathway are semi-permissive to Tcf-viruses replication. I showed that this semi-permissiveness impacts mainly on the activity of the E2 promoter. To increase the viral toxicity, I used a viral backbone that retains the wild type E2 promoter to express a prodrug converting enzyme. I showed that this results in increased cytotoxicity in colon cancer cells upon addition of the prodrug.

Viruses with multiple mutations are safer because more than one recombination event is needed in order to revert to the wild type sequence. Thus, it is interesting to look for a new Tcf-E2 promoter that will enable proper E2 expression in the semi-permissive cell lines. With respect to this issue, the ABFT34 virus should allow us screening for new promoters in the context of a viral infection. Alternatively, the AHB23'4 virus contains an E1A-HD2 fusion gene that might further activate transcription from Tcf-containing promoters and thus bypass the higher activation signal that is apparently required by the E2 promoter.

The molecular mechanism of the semi-permissivity is a potentially very interesting and useful question to address. Semi-permissivity might be the consequence of nuclear β-catenin export by APC. In that case, this indicates that the vast majority of colorectal cancers would be permissive to the Tcf-viruses, since most of them have APC mutations that abolish its export function. On the other hand, specific inhibitors of the Wnt pathway might be differentially expressed in the various cell lines tested. If this is the case, inhibiting these proteins might be a way to increase Wnt activity, provided it does not lead to stabilisation of β-catenin.

Protein inhibition can be achieved for example by expression of a dominant-negative protein, or expression of a peptide that binds to the active site or prevents protein-protein interaction. Inhibition of protein expression can be achieved *via* RNAi-mediated silencing. In that respect, it

might be possible to incorporate an shRNA into the PolIII-encoded VA RNAs. These RNAs are attractive because the termination site is very precise. Preliminary data show that it is formally possible to add a functional shRNA into the sequence of the VAI RNA (S.Pébernard, pers. comm.). If this approach proves to be efficient, the question of the target will remain. One can target specific inhibitors of the Wnt pathway, or genes that maintain the cellular architecture and prevent efficient viral particles release such as lamin. Other potential targets are genes involved in drug resistance or in the degradation of therapeutic drugs like MDR (Multi-Drug Resistance) and DPD (DihydroPyrimidine Dehydrogenase), respectively.

The E4 promoter behaved roughly like the wild type promoter. A tighter E4 promoter will probably increase the active repression in non-permissive cells, and thus lead to more selective viruses. I deleted the entire wild type control region of the Tcf-E4 promoter and reporter assays showed that this promoter is more selective for the Wnt pathway than the previous Tcf-E4 promoter (data not shown). To check the activity of this promoter in the context of a virus, it was inserted into an AB4 background.

A way to check if the yCD sequence contains a cryptic enhancer that activates nearby promoters is to replace yCD by another suicide gene like the *E.coli* nitroreductase gene. Similarly, deletion of the E4orf7 gene should prevent transactivation of the E2 promoter by E4orf6/7. Another possibility would be to incorporate the new E4 promoter into one of the yCD-viruses to see whether E4 protein expression will be abolished in normal cells.

Finally, it is known that the tumour core or the tumour endothelium is often hypoxic. One way to enable our viruses to spread in the tumour endothelium or to further activate the virus in the tumour core would be to insert a hypoxia-responsive element in the Tcf-E1A and Tcf-E4 promoters. I propose to create dual Tcf/HRE promoters that will be responsive to both the Wnt pathway and to hypoxia. The activity of such a promoter in different conditions will be analysed by reporter assays. Of particular interest is the activity of the promoter in hypoxic cells with an inactive Wnt pathway (like the tumour endothelium cells). Provided both pathways are not mutually exclusive, Tcf/HRE promoters could be more efficient than the actual Tcf promoters to treat colon cancer *in vivo*.

4. Annexes

4.1. Abbreviations

5-FC	5-Fluorocytosine
5-FU	5-Fluorouracil
Ad	Adenovirus
ADP	Adenovirus death protein
AP-1	Activator protein 1 (jun)
APC	Adenomatous polyposis coli
ara-C	Cytosine arabinoside
Arm	Armadillo
ATF	Activating transcription factor
BCL9	B-cell lymphoma 9 protein
BRE	TFIIB responsive element
β-TRCP	β-Transducin repeats containing protein
CAR	Coxsackie and Adenovirus receptor
CD / yCD	Cytosine deaminase / yeast Cytosine deaminase
CHX	Cycloheximide
CIN	Chromosomal instability
CKI	Casein kinase I
CMV	Cytomegalovirus
CPE	Cytopathic effect
CR1 to CR4	Conserved regions 1 to 4
CRC	Colorectal cancer
CREB	cAMP response element binding protein
CtBP	C-terminal binding protein
DBP	single-stranded DNA binding protein
DIX	Domain present in dishevelled and axin (formerly DAX)
DLG	Discs large homolog
DNA PK	DNA proteine kinase
Dsh	Dishevelled (cf. Mad)
E1A, E1B, E2, E3, E4	Early unit 1A, 1B, 2, 3, and 4
E2F	E2 factor
E4F-1	E4 factor 1
eIF-2α	Eukaryotic translation initiation factor 2α
eIF4F	Eukaryotic translation initiation factor 4F (eIF4A, 4E, and 4G)
EMCV	Encephalomyocarditis virus
FAP	Familial adenomatous polyposis
fopflash	False Tcf-optimal promoter driving luciferase
FRAT	Frequently rearranged in advanced T-cell lymphomas
Fz	Frizzled
GCV	Gancyclovir
GSK3β	Glycogen synthase kinase 3β
HAT	Histone acetyltransferase
HD2	Homology domain 2
HLF	Human lung fibroblasts
hNHeps	Human normal hepatocytes
HMG	High mobility group
HNPCC	Hereditary non-polyposis colorectal cancer
HRE	Hypoxia-responsive element
HSV	Herpes simplex virus
IFN	Interferon
icat	inhibitor of β-catenin
IRES	Internal ribosome entry site

ITR	Inverted terminal repeat
L1 to L5	Late unit 1 to 5
LRP	Low-density lipoprotein receptor-related protein
MCR	Mutation cluster region
MLP	Major late promoter
MLTU	Major late transcription unit
m.o.i.	Multiplicity of infection
NES	Nuclear export signal
NF-κB	Nuclear factor - κB
NHD	N-terminal homology domain
NLS	Nuclear localisation signal
Ntr	Nitroreductase
Pol	Polymerase
RGD	Arginine – Glycine – Aspartic acid
p300 / CBP	300kDa protein / CREB-binding protein
p400	400kDa protein part of the TIP60 complex
PAR	Ser/Thr protein kinase (partitioning)
P/CAF	p300/CBP associated factor
PCR	Polymerase chain reaction
pfu	Plaque-forming units
PI3K	Phosphatidylinositol 3-kinase
PDZ	protein-protein interaction domain, target to sub-membranous sites
PKR	double-stranded RNA-activated protein kinase R
PML	Promyelocytic Leukaemia protein
POD	PML oncogenic domain
polyA signal	Polyadenylation signal
PP2A	Protein phosphatase 2 A
pRb	Retinoblastoma protein
(p)TP	(pre) Terminal protein
Pygo	Pygopus
RACE	Rapid amplification of cDNA ends
RID	Receptor internalisation and degradation protein
RING-finger	domain involved in the mediation of ubiquitin ligase activity
RGS	Regulator of G-protein signalling
RNAi	RNA interference
Rpd3	Reduced potassium dependency protein 3 (histone deacetylases)
RSV	Rous sarcoma virus
RT	Reverse transcription
SAEC	Small airways epithelial cells
SAMP	Serine – Alanine – Methionine – Proline domain
SCF	Skp1 / Cullin / F-Box complex
SWI/SNF	DNA remodelling complex
TAF	TBP-associated factor
TBP	TATA binding protein
TCF/LEF	T-cell transcription factor / Lymphoid enhancer binding factor
TFIIB	Transcription initiation factor IIB
TFIID	Transcription initiation factor IID
tk	thymidine kinase
TNF	Tumour necrosis factor
topflash	Tcf-optimal promoter driving luciferase
TRRAP	Transformation/transcription domain-associated protein
UTR	Untranslated region
VA RNA	Virus-associated RNA
Wnt	Wingless – int(egration)

4.2. Oligos list

oCF1   5'–GGGTGGAAAGCCAGCCTCGTG–3'
oCF2   5'–ACCCGCAGGCGTAGAGACAAC–3'
oCF3   5'–TCCAGATCAAAGGGATTAAGATCAAAGGGCCACCACCTCATTAT–3'
oCF4   5'–TCCCTTTGATCTCCAACCCTTTGATCTAGTCCTATTTATACCCG
        GTGA–3'
oCF5   5'–TCCCTTTGATCTCCACTAGTGTGAATTGTAGTTTTCTTAAAATG–5'
oCF6   5'–GAACTAGTAGTAAATTTGGGCGTAACC–3'
oCF7   5'–ACGCTAGCAAAACACCTGGGCGAGT–3'
oCF8   5'-CATTTTCAGTCCCGGTGTCG–3'
oCF9   5'-ACCGAAGAAATGGCCGCCAG–3'
oCF10   5'–TCTGTAATGTTGGCGGTGCAGGAAG–3'
oCF12   5'-ATGGCTAGGAGGTGGAAGAT–3'
oCF13   5'-GTGTCGGAGCGGCTCGGAGG–3'
oCF17   5'-CGACGCCTATATATCTAGTCCTTAAGA–3'
oCF18   5'–CTAGTCCTTAAGAGCTAGTCCTTAAGAGTCAGC–3'
oCF19   5'–ATCAAAGGGTTGGAGATCAA–3'
oCF20   5'-CATATGGCTAGCTAAGCGATCAAAGGGTTGGAG–3'
oCF21   5'-CATTGCCCTTTGATCTCCTACTGAACCCTTTGATCG–3'
oCF22   5'–CATAAGATCAAAGGGACTGTAGACAGATCAAAGGGACTAGTG
        CCATTATGAGCAAGG–3'
oCF23   5'–CTAGTACGTTGTTATTTGTTTTTTTCG–3'
oCF24   5'-CTAGCGAAAAAAACAAATAACAACGTA–3'
oCF27   5'-AGTCCCTTTGATCTTAATCCCTTT–3'
oCF28   5'-AGTCCATTATGAGCAAGGAAATT–3'
oCF31   5'–TCGCTAGCCAGGCACAATCTTCGCATTTCTTTTTTTCCAGATGG
        TGACAGGGGGAATGGC–3'
oCF32   5'–TGACTAGTTATTCACCAATATCTTCAAA–3'
oCF35   5'-CTAGTTTTCGCGCCTGCGTTTTCGCGCG–3'
oCF36   5'-CTAGCGCGCGAAAACGCAGGCGCGAAAA–3'
oCF46   5'–ATACTATACGAAGTTATCGCCATTATGAGCAAGG–3'
oCF47   5'–ACATTATACGAAGTTATCTCGAGTTAACCTAGTCCTTAAGAGTCA–3'
oCF48   5'–ATGCTATACGAAGTTATTTAGCCCAAGAGCAACA–3'
oCF49   5'–ACATTATACGAAGTTATCGGCGGCGGCTGCTTGG–3'
oCF61   5'-TTCCTCGCATGGTGGTGA–3'
oCF62   5'–GAAAGAAGGTCCCTGTGTATTTG–3'
oCF63   5'–GGGCAAGGCCGGTCTG–3'
oCF64   5'–GTGAGTATGCAGGGCCCTGAGTAT–3'
oCF65   5'-GATGGCCAATAAAGCTGCAG–3'
oCF66   5'-CTGTGTGTTCAGAGGCGCTG–3'
oCF67   5'–AGTTTCTTTATTCTTGGGCAATGT–3'
oCF68   5'–AGTCGTTTGTGTTATGTTTCAAC–3'
oCF69   5'–ATGCTAGCGAATTCCGCCCCTCTC–3'
oCF70   5'–ATACTAGTTatgCATATTATCATCGTGTTT–3'
oCF71   5'–GTGACAGGGGGAATGGCAAG–3'
oCF76   5'–TGACTAGtTTATTCACCAATATCTTCAAA–3'
oCF77   5'–GGAATTCGCTAGTTTCTCTACTCTTGGGCAATGTA–3'
oCF83   5'–CGGGCCTGGGGCGTTTACAG–3'
oCF84   5'–GGTAAGGTGTAAACCTGTGATTGC–3'
oCF85   5'–TACCCGGGAGCAATAGCTCTTCAG–3'
oCF86   5'-ACCCCGGGATTCTGCTGCGGTCCCCAC–3'
oCF109 5'–TTGTGATGAGTCTTCCT–3'
oCF110 5'–CTGCTCCTCTTCCCGACT–3'
oKH9   5'–GGTGGTGGAGATGCTAAACTCACTTGGTC–3'
G76   5'– CGGAATTCAAGCTTAATTAACATCATCAATAATATACC–3'

Sequencing plasmids
IF171   5'–AGTTGCTCTGCCTCTCCAC–3'
IF272   5'–GCCATTAATGCAGGAGATG–3'
IR110   5'–CATCTCTACAGCCCATAC–3'
IR190   5'–TGTCTGAACCTGAGCCTGAG–3'
IR213   5'–CAGGTCCTCATATAGCAAAGC–3'
IR215   5'–CGTGATTAAAAAGCACCACC–3'
IR281   5'–GGAGAAAGGACTGTGTACTC–3'
M13fwd 5'–AGGGTTTTCCCAGTCACGACGTT–3'
M13rev 5'–AGCGGATAACAATTTCACACAGGA–3'

4.3.   Plasmids list

Name	Resistance	Backbone	Construction
pCF1	Chr	pNKBAC39	pMB19·PacI and self-ligated (Gagnebin 1999)
pCF4	AmpTrp1	pLS77	Ad5 left terminus (nt 1 to 952) PCR'ed with G76/oCF1·PacI into pMB1·PacI/BamHI^{Kf} (pMB1 is based on pLS77)
pCF6	AmpTrp1	pLS77	Ad5 right terminus (nt 35369 to 35938) PCR'ed with G76/oCF2·PacI into pMB1·PacI/BamHI^{Kf}
pCF9	AmpNeo	pcDNA3	wt E1A 12S into pcDNA3 (Alevizopoulos 1998)
pCF10	AmpNeo	pcDNA3	E1A 12S mutant (124A-135A, unable to bind pRb) into pcDNA3 (Alevizopoulos 1998)
pCF12	AmpNeo	pcDNA3	E1A 12S mutant (Δ26-35, unable to bind p400) into pcDNA3 (Alevizopoulos 1998)
pCF16	AmpTrp1	pLS77	iPCR of pCF6 with oCF3/oCF5. E4 with Tcf b. sites
pCF17	Amp	pGL3-Basic	pMB39 (wtE2·wtE3, E2-driven luc, Brunori 2001)
pCF20	Amp	pGL3-Basic	pMB59 (mutE2·wtE3, E2-driven luc, Brunori 2001)
pCF21	Amp	pGL3-Basic	pMB60 (mutE2·mutE3, E2-driven luc, Brunori 2001)
pCF25	Amp	pLS77	iPCR of pCF4 with oCF3/oCF4 to give pCF13. pCF13·PvuII into pCF4 to get rid of the point mutation in E1A 12S
pCF27	Amp	pCMV-HA	p300 in pCMV-HA
pCF32	AmpNeo	pcDNA3	E1A 12S mutant (Δ2-11, unable to bind p300) from a pBH2-based vector (Alevizopoulos 1998)·BamHI·EcoRI into pCF12
pCF34	AmpTrp1	pLS77	PCR of packaging signal with oCF6/oCF7·SpeI·NheI into pCF16
pCF37	Amp	pcDNAI	ΔN-βcatenin dominant mutant (van de Wetering 1997)
pCF42	Amp	pGL3-Basic	pCF17·AflII·SacI^{T4}, "minimal" E2=E3 luc reporter
pCF43	AmpNeo	pcDNA3	E1A 12S mutant (Δ64-68, unable to bind p300) from a pBH2-based vector (Alevizopoulos 1998)·BamHI·EcoRI into pCF9
pCF45	AmpNeo	pcDNA3	iPCR of pCF9 with oCF10/12. E1A 12S ΔCR1
pCF46	AmpTrp1	pLS77	pCF56·HindIII^{Kf}·SalI into pCF34·PstI^{T4}·SalI. Pre gap-repair vector: mutant left ITR Δ2-11 / mutant right ITR
pCF52	AmpTrp1	pLS77	pCF61·HindIII^{Kf}·SalI into pCF6·PstI^{T4}·SalI. Pre gap-repair vector: wt left ITR Δ2-11 / wt right ITR
pCF56	Amp	short-pLS77	iPCR of pRDI-284 (pCF25-derived) with oCF8 and oCF9. Mutant left ITR E1A Δ64-68
pCF61	Amp	short-pLS77	iPCR of pRDI-283 (pCF4-derived) with oCF8 and oCF9. Wild type left ITR E1A Δ2-11
pCF78	Chr	pNKBAC39	pRDI-285·PacI into pCF1. Gap repair vector. mut left ITR / wt E1A / mut right ITR
pCF79	Chr	pNKBAC39	pCF46·PacI into pCF1. Gap repair vector. mut left ITR / E1A Δ2–11 / mut right ITR

pCF81	Chr	pNKBAC39	pCF52·PacI into pCF1. Gap repair vector. wt left ITR / E1A Δ2–11 / wt right ITR
pCF83	Amp	pGL3-Basic	PCR of pCF4 with G76/oCF13·HindIII·PNK into pCF26·NcoI^{Kf}·HIII. Wild type E1A promoter driving luciferase
pCF86	Amp	pGL3-Basic	PCR of pCF25 with G76 and oCF13·HindIII·PNK into pCF26·NcoI^{Kf}·HIII. Mutant E1A promoter driving luciferase
pCF88	AmpNeo	pcDNA3	E1A 12S ΔC52 (K. Alevizopoulos)
pCF89	AmpNeo	pcDNA3	E1A 12S E55, P/CAF binding def. mut. (Reid 1998)
pCF91	AmpNeo	pcDNA3	E1A 12S LDLA4, CtBP binding deficient mutant (K. Alevizopoulos)
pCF93	Amp	pCX	Flag-tagged P/CAF (Blanco 1998)
pCF94	Amp	pCX	Flag-tagged P/CAF ΔHAT (Δ579-608) (Blanco 1998)
pCF102	AmpNeo	pcDNA3	pCF89·BstXI into pCF32·BstXI. E1A Δ2-11 and E55
pCF105	Amp	pBSK+CβS	(CMV/β-globin/splice)-Flag-Tip49 (Wood 2000)
pCF106	Amp	pBSK+CβS	CβS-Flag-Tip49 DN (Wood 2000)
pCF107	Amp	pcDNA1	myc TAG–βcat (TAD)–hTcf1 (Korinek 1997)
pCF109	Amp	pcDNA1	hTcf4 (Korinek 1997)
pCF110	Amp	pRc-CMV	pRc12S (Ad2 E1A 12S) from René Bernards
pCF111	Amp	pRc-CMV	pRc13S (Ad2 E1A 13S) from René Bernards
pCF113	Amp	pcDNA3	pCF9·BstXI -> Gpur of 5.4, 0.68 and 0.32 kb. 0.68 kb·BsmBI -> Gpur of 0.54 kb. pCF111·BstXI·BsmBI -> Gpur of 0.27 kb (CR3) 4-way lig of 5.4, 0.54, 0.32 and 0.27 => E1A 13S vector
pCF118	Amp	pGL3-Basic	iPCR of pCF21 with oCF17/oCF19. New TATA and BRE upstream of E2-Tcf wt TATA
pCF122	Amp	pGL3-Basic	iPCR of pCF21 with oCF18/oCF19. Old TATA and BRE upstream of E2-Tcf wt TATA
pCF127	Amp	pGL3-Basic	iPCR of pCF21 with oCF17/oCF20. New TATA, BRE, and spacer upstream of E2-Tcf wt TATA
pCF128	Amp	pGL3-Basic	iPCR of pCF21 with oCF18/oCF20. Old TATA, BRE, and spacer upstream of E2-Tcf wt TATA
pCF132	Amp	pGL3-Basic	iPCR of pCF118 with oCF21/oCF22. New TATA and BRE. 20 bp phasing btw Tcf sites + SpeI site.
pCF137	Amp	pGL3-Basic	iPCR of pCF118 with oCF27/oCF28. New TATA and BRE. 15 bp phasing btw Tcf sites + SpeI site.
pCF144	Amp	pGL3-Basic	iPCR of pCF122 with oCF27/oCF28. Old TATA and BRE. 15 bp phasing btw Tcf sites + SpeI site.
pCF157	Amp	pUC19	wtAd5·KpnI·XbaI (25838-28592) into pUC19
pCF159	Amp	pUC19	wtAd5·KpnI·XbaI (30470-33598) into pUC19 (rev)
pCF162	Amp	pGL3-Basic	iPCR of pCF21 with oCF21/oCF22. E2-Tcf wt basal promoter with Tcf sites spaced by 20bp + SpeI
pCF164	Amp	pGL3-Basic	iPCR of pCF127 with oCF21/oCF22. New Basal, spacer, 20bp phasing between Tcf sites and SpeI
pCF167	Amp	pGL3-Basic	iPCR of pCF128 with oCF21/oCF22. Old Basal, spacer, 20bp phasing between Tcf sites and SpeI
pCF169	Amp	pGL3-Basic	iPCR of pCF21 with oCF27/oCF28. E2-Tcf wt basal promoter with a SpeI site upstream of Tcf sites
pCF171	Amp	pGL3-Basic	iPCR of pCF128 with oCF27/oCF28. New Basal, spacer, 15bp phasing between Tcf sites and SpeI
pCF174	Amp	pGL3-Basic	oCF23/oCF24 into pCV20·SpeI. 2 FoxM1 sites fwd
pCF175	Amp	pGL3-Basic	oCF23/oCF24 into pCV20·SpeI. 1 FoxM1 site fwd
pCF176	Amp	pGL3-Basic	oCF23/oCF24 into pCV21·SpeI. 2 FoxM1 sites fwd
pCF177	Amp	pGL3-Basic	oCF23/oCF24 into pCV21·SpeI. 1 FoxM1 site fwd
pCF180	Amp	pGL3-Basic	oCF23/oCF24 into pCV26·SpeI. 2 FoxM1 sites fwd
pCF181	Amp	pGL3-Basic	oCF23/oCF24 into pCV26·SpeI. 1 FoxM1 site fwd
pCF186	Amp	pGL3-Basic	oCF35/oCF36 into pCV26·SpeI. 1 E2F-1 site fwd
pCF188	Amp	pGL3-Basic	oCF35/oCF36 into pCV26·SpeI. 1 E2F-1 site rev
pCF189	Amp	pGL3-Basic	oCF35/oCF36 into pCV21·SpeI. 1 E2F-1 site fwd

pCF193	Amp	pGL3-Basic	pCV26·NheI^{Kf}·NdeI^{Kf} self-lig (-6 bp *vs.* pCF26)
pCF194	Amp	pGL3-Basic	pCV26·NheI^{Kf}·NdeI^{Kf} self-lig (-4 bp *vs.* pCF26)
pCF195	Amp	pGL3-Basic	pCV26·NheI^{Kf}·NdeI^{Mung} self-lig (+4 bp *vs.* pCF26)
pCF196	Amp	pGL3-Basic	pCV26·NheI^{Kf}·NdeI^{Mung} self-lig (+3 bp *vs.* pCF26)
pCF197	Amp	pGL3-Basic	pCV26·NdeI^{Kf}·NheI^{Mung} self-lig (-8 bp *vs.* pCF26)
pCF199	Amp	pGL3-Basic	pCV26·NdeI^{Kf}·BlpI^{Mung} self-lig (-11 bp *vs.* pCF26)
pCF206	AmpNeo	pcDNA3	yCD PCR'ed from yeast with oCF31/32·SpeI·NheI into pcDNA3·XbaI (mut)
pCF231	AmpNeo	pcDNA3	yCD PCR'ed from yeast with oCF31/32·SpeI·NheI into pcDNA3·XbaI (mut)
pCF232	Amp	pcDNA3	pCF231·ApaI^{T4}·BamHI into pCF206·KpnI^{T4}·BamHI. yCD wt
pCF234	AmpPuro	pUC19	pES126·HindIII^{Kf}·BamHI (TET-VP16 TAD) into pRDI292·SmaI·BamHI. EF1α-TET-VP16/SV40/puro
pCF237	AmpUra3	pRS406	iPCR of pMB66 (mut E3/4·Tcf-E2 pop-in/out) with oCF46/oCF47 -> LoxP511 before the E2 promoter
pCF241	Amp	pUC19	iPCR of pCF157 with oCF48/oCF49 -> LoxP into 33k intron
pCF243	AmpPuro	pUC19	Lentivirus : EF1α promoter and SV40/puro cassette (pRDI-292)
pCF247	AmpBleo	pUC19	pBabeBleo·EcoRI·ClaI into pRDI-282·EcoRI·BstBI. EF1α/BamHI·EcoRI//SV40/Bleomycin lentivirus
pCF248	AmpNeo	pUC19	pBabeNeo·EcoRI·ClaI into pRDI-282·EcoRI·BstBI. EF1α/BamHI·EcoRI//SV40/Neomycin lentivirus
pCF249	AmpUra3	pRS406	pES149·StuI·XhoI (Tet-RE) into pCF237·HpaI·XhoI –> LoxP511-TET-E2 promoter in popin/out vector
pCF251	Amp	pBSII SK(-)	hPygo1 in pBSKII SK(-) (Kramps 2002)
pCF252	Amp	pBSII SK(-)	hPygo2 in pBSKII SK(-) (Kramps 2002)
pCF253	AmpUra3	pRS406	pCF241·KpnI^{T4}·AflII into pCF249·SgrAI^{Kf}·AflII. LoxP in 33k intron + LoxP511-TET-E2 promoter in pop inout vector
pCF255	AmpPuro	pUC19	pCF251·XhoI^{Kf}·EcoRI into pCF243·SmaI·EcoRI. EF1α/hPygo1//SV40/puro lentivirus
pCF256	AmpNeo	pUC19	pCF252·XhoI^{Kf}·BamHI into pCF248·SmaI·BamHI. EF1α/hPygo2//SV40/neo lentivirus
pCF260	AmpUra3	pRS406	pCF204(Fibre K7 in pUC19)·KpnI·XbaI into pMB31. Fibre K7 in popin/out vector
pCF262	Amp	pUC19	iPCR of pCF159 with oCF67/68. SpeI site 3' of fibre
pCF267	Amp	pUC19	PCR of IRES from pSE280-IRES with oCF69/oCF70·NheI·SpeI into pCF262·SpeI. Fibre-IRES (mut, duplication at beg. of IRES)
pCF270	Amp	pUC19	yCD PCR'ed from pCF232·NheI·SpeI into pCf262·SpeI. Fibre-yCD (PolyA not optimal)
pCF271	Amp	pGL3-Basic	pCF249·EcoRI·AflII into pCF17. wtE3-TET-E2 luc
pCF272	Amp	pUC19	pCF260·BstXI·XbaI into pCF159. K7 fiber
pCF274	Amp	pUC19	pCF267·BstXI·XbaI into pCF272. Fibre-IRES (mut)
pCF276	Amp	pUC19	pCF270·BstXI·SpeI into pCF272. Fibre-yCD
pCF277	Amp	pUC19	pCF262·BstXI·SpeI into pCF272. wtFiber – SpeI
pCF278	Amp	pUC19	pCF265(pCF267-K7)·BstXI·SpeI into pCF272
pCF281	Amp	pUC19	yCD PCR'ed from pCF232 with oCF31/oCF76·NheI·SpeI into pCF272·SpeI. K7-splice-yCD
pCF284	Amp	pUC19	yCD PCR'ed from pCF232 with oCF72/oCF76·PstI·SpeI into pCF266 (pUC19-like). yCD (PstI/SpeI)
pCF291	Amp	pUC19	pCF284·PstI^{T4}·SpeI into pCF274·BfrBI·SpeI. wt fibre-IRES-yCD (Poly(A) after fibre)
pCF298	Amp	pUC19	pCF281·AflII·SpeI into pCF276. Fibre-splice-yCD
pCF299	Amp	pUC19	delete Poly(A) of fibre in pCF274. 3-way ligation. 5.1 kb (AflII/BstXI) frag of pCF291 + 0.9 kb (AflII/EcoRI) frag of pCF274 + oKH9/oCF77 (RI/BXI).

pCF302	Amp	pUC19	delete Poly(A) of fibre in pCF278. 3-way ligation. 5.1 kb (AflII/BstXI) frag of pCF291 + 0.9 kb (AflII/EcoRI) frag of pCF278 + oKH9/oCF78 (RI/BXI).
pCF308	Amp	pUC19	yCD PCR'ed from pCF284·SpeI into pCF278·BfrBI·SpeI
pCF315	Amp	pUC19	pCF302·EcoRI into pCF299. Fiber-NoPoly(A)-IRES.
pCF317	Amp	pUC19	pCF308 PCRed with oCF69/79, used as primer with oCF76 (2nd PCR). Re-amplified with oCF69/76. Cut with DraIII into pCF308.
pCF325	AmpUra3	pRS406	pCF298·XbaI·SpeI into pCF260. Fiber-splice-CD in pop in/out vector
pCF328	AmpUra3	pRS406	pCF315·XbaI·SpeI into pCF260. Fiber-IRES in pop in/out vector
pCF330	AmpUra3	pRS406	pCF317·AvrII·SpeI into pCF328. Fiber-IRES-CD in pop in/out vector.
pCF331	Amp	pBSII SK(-)	hBCL-9 in pBSII SK(-) (Kramps 2002)
pCF343	AmpPhleo	pUC19	pCF331·NdeI into pCF258·NdeI (mutant BCL9 in a lentivirus). EF1α/hBCL9//SV40/phleo lentivirus
pCF357	Amp	pUCDNA3	pUC19·HindIII·EcoRI into pcDNA3. Extended MCS in pcDNA3.
pCF358	Amp	pUCDNA3	vpCF2·SpeI^{Kf}·KpnI into pCF357·EcoRI^{Kf}·KpnI. Leftmost part of vCF02 (1-1748) into pUCDNA3. Tcf-E1A and Tcf-E1B.
pCF387	Amp	pUCDNA3	pALP8 (E1A 13S-HD2)·BsmBI·HpaI into pCF358. vpCF2-HD2 to make gap repair.
pCF389	Amp	pLS77	pCF358·SphI into pCF34. Pre-gap repair. Tcf-E1A, Tcf-E1B // Tcf-E4 (classical Tcf-E4).
pCF393	Amp	pLS77	pCF387·KpnI·XhoI into pCF389. Pre-gap repair. Tcf-E1A(HD2), Tcf-E1B // Tcf-E4 (classical Tcf-E4)
pCF398	Chr	pNKBAC39	pCF393·PacI into pCF1. Gap repair vector.Tcf-E1A (HD2), Tcf-E1B // Tcf-E4 (classical E4)

Constructed by Christelle Volorio

Name	Resistance	Backbone	Construction
pCV1	Amp	pGL3-basic	pCF6·HindIII, purified, AluI (E4wt promoter) into pGL3-Basic·NcoI^{Kf}·HindIII
pCV2	Amp	pGL3-basic	pCF16·HindIII, purified, AluI (E4wt promoter) into pGL3-Basic·NcoI^{Kf}·HindIII
pCV3	Amp	pGL3-basic	pCF34·HindIII, purified, AluI (E4wt promoter) into pGL3-Basic·NcoI^{Kf}·HindIII
pCF13	Amp	pGL3-basic	pCF118·NarI·KpnI into pCF21
pCV15	Amp	pGL3-basic	pCF122·NarI·KpnI into pCF21
pCV17	Amp	pGL3-basic	pCF127·NarI·KpnI into pCF21
pCV19	Amp	pGL3-basic	pCF128·NarI·KpnI into pCF21
pCV20	Amp	pGL3-basic	pCF132·NarI·KpnI into pCF21
pCV21	Amp	pGL3-basic	pCF137·NarI·KpnI into pCF21
pCV23	Amp	pGL3-basic	pCF144·NarI·KpnI into pCF21
pCV25	Amp	pGL3-basic	pCF162·NarI·KpnI into pCF21
pCV26	Amp	pGL3-basic	pCF164·NarI·KpnI into pCF21
pCV27	Amp	pGL3-basic	pCF167·NarI·KpnI into pCF21
pCV29	Amp	pGL3-basic	pCF169·NarI·KpnI into pCF21
pCV30	Amp	pGL3-basic	pCF171·NarI·KpnI into pCF21

Constructed by Anne-Laure Pittet

Name	Resistance	Backbone	Construction
pALP1	Amp	pUC19	pCF9·BamHI·EcoRI into pCF153 (wtAd5 (14290-15376)·KpnI). E1A 12S in pUC19
pALP2	Amp	pUC19	pCF113·BamHI·EcoRI into pCF153 (wtAd5 (14290-15376)·KpnI). E1A 13S in pUC19
pALP3	Amp	pUC19	iPCR of pALP1 with oCF85/oCF86. E1A/XmaI site in pUC19
pALP5	Amp	pUC19	PCR of hBCL9(HD2) from pCF250 (like pCF331 but w/mutation at the C-term) with primers oCF85/oCF86·XmaI into pALP3
pALP7	Amp	pUC19	pALP5·PshAI·NdeI into pALP2. E1A 13S-HD2 in pUC19
pALP8	Amp	pcDNA3	pALP7·HpaI·BamHI into pCF113. E1A 13S-HD2 in pcDNA3
pALP9	Amp	pcDNA3	pALP5·HpaI·BamHI into pCF9. E1A-HD2 in pcDNA3

4.4. Contributions

I would like to specifically acknowledge the work that was done by my diploma students and that has been incorporated into this thesis, and I will briefly indicate the projects in which the students were involved, as well as the figures they made, or for which they participated.

Christelle Volorio helped me in characterising my first generation of viruses (Figure 2.3b). She also did numerous experiments with the E4 promoters (Figure 2.6), the E1A13S protein (Figure 2.8), and semi-permissivity (Figure 2.6). Anne-Laure Pittet was involved in the BCL9/Pygopus story (Figure 2.13, Figure 2.14), did time-course infection experiments (Figure 2.16, Figure 2.17), and finally constructed and analysed the E1A-HD2 fusion proteins (Figure 2.24). Christophe Hug did experiments with the latest E4 promoters (data not shown) and MTT asays with the yCD viruses (data not shown). Finally, Jessica Vazquez is doing RNA interference against APC (data not shown), constructing a new generation of viruses, and will help me in characterising the new E4 viruses.

I am deeply indebted to these people, and would like again to thank them for all the work they did.

5.   References

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