**The Dissertation Committee for Gail Perry Smith Certifies that this is the approved version of the following dissertation:**

# Mouse Mammary Tumor Virus activates Cdc42 leading to filopodia formation and transformation of mammary epithelial cells

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# Mouse Mammary Tumor Virus activates Cdc42 leading to filopodia formation and transformation of mammary epithelial cells

**by**

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# Dedication

To my husband and my children for their love and support.

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**Mouse Mammary Tumor Virus activates Cdc42 leading to filopodia formation and transformation of mammary epithelial cells**

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Mouse mammary tumor virus (MMTV) is a B type retrovirus characterized by assembly of viral "A" particles in the cytoplasm that move to the membrane where they acquire an envelope and exit the cell. Viral exit is associated with reorganization of cortical actin and accumulation of virus in filamentous projections at the cell surface. Here we show that MMTV egress and exit from the cell depends on actin polymerization mediated by the activation of Cdc42 and WASP. Cytochalasin B treatment blocked both accumulation of virus in filopodia and viral exit. Active virus production correlated with accumulation of activated Cdc42 and MMTV Gag on immobilized Pak columns. Both Cdc42 and WASP co-localized with virus particles in the cytoplasm and at the cell surface. Alternatively, dominant-negative Cdc42 or a mutant WASP construct without

the Cdc42-binding domain failed to co-localize with virus and prevented MMTV-induced cytoskeletal reorganization as well as viral egress and exit. Infection with virus conferred on these cells the ability to grow in serum-free media, to grow on soft agar and to form foci, features indicative of transformation. Uninfected cells expressing constitutively active Cdc42 exhibited similar characteristics. Furthermore, dominant negative Cdc42 blocked transformation by MMTV. These findings suggest that activation of the Cdc42/WASP/ARP2/3 pathway is required for viral exit and that viral activation of Cdc42 is both required and sufficient to cause transformation.

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# Chapter 1: Mouse mammary tumor Virus (MMTV)

General characterization of MMTV

Retroviruses are RNA viruses that infect host cells, integrate into the cellular genome, and co-opt the cellular machinery to carry out their life cycle. During their life history, the virions utilize the host cytoskeleton to facilitate their dispersal into adjoining cells[1]. The concomitant viral infection of the adjacent cells can result in the transformation of those cells and the formation of tumors in the host tissues. Many tumor causing retroviruses have oncogenes as a component of their proviral DNA[2]. Other retroviruses cause tumor formation via cis-activation of host genes upon the insertion of proviral DNA into the host genome [3].

Mouse mammary tumor virus (MMTV) is classified as a B-type retrovirus but is the only representative of that category. The virus can be transmitted endogenously when embryonic germ line cells are infected[4] but it is usually transmitted exogenously to infant mice via infectious mature virus in the infected mother's milk[5]. The virions initially infect the B-lymphocytes of infant mice located in the Peyer's patches of the gastrointestinal tract [6]. Virus expression results in production and cell surface display of a superantigen protein, SAg that is encoded in the MMTV genome. The Sag binds both MHC on B cells and the Vβ of the T cell receptor making it a potent T cell stimulus [7]. The stimulated T-cells then cause the proliferation of many B-lymphocytes that subsequently also become infected with MMTV [8]. These secondary-infected B-lymphocytes carry the MMTV virions to the mammary gland where the virus infects the

mammary epithelial cells [9]. The chronic infection of mammary cells induces the formation of malignant tumors, mammary adenocarcinomas. Infected T-cells rarely become tumorigenic; though, when T-lymphocytes are transformed, T-cell lymphomas do occur [10] [11] [12].

Structure of MMTV

The mature MMTV virion, or B particle, is an enveloped structure that is 100nm in diameter. Envelope proteins are composed of a transmembrane glycoprotein protein, TM, and a surface glycoprotein, SU, that are located on the surface of the viral lipid envelope. Beneath the lipid envelope, there are three structural proteins; the MA (matrix), the CA (capsid), and the NC (nucleocapsid) proteins [13]. The MA protein is located directly beneath the lipid bilayer; while, the CA (capsid), and NC (nucleocapsid) proteins comprise the core of the virion. The core of the virion is an eccentric, spherical structure made up of the CA protein that forms a shell around the viral genome. Within the capsid shell, the NC protein is bound to the viral genomic RNA; in addition, there are several enzymatic proteins located within the core of the virion. The viral protease, PR , the reverse transcriptase, RT, and the integrase, IN are found within the capsid shell of the core of the virus [14]. Deep within the core associated with the nucleocapsid protein, NC is the viral genome made up of two strands of RNA. Both strands are identical and they are 8,588 bases in length. They are of positive polarity and they have a 5' 7-methylguanosine cap and a 3' poly A tail. In addition to the RNA genome there is also a host tRNA, lys-3, incorporated into the core of the virion [15] (Figure 1).

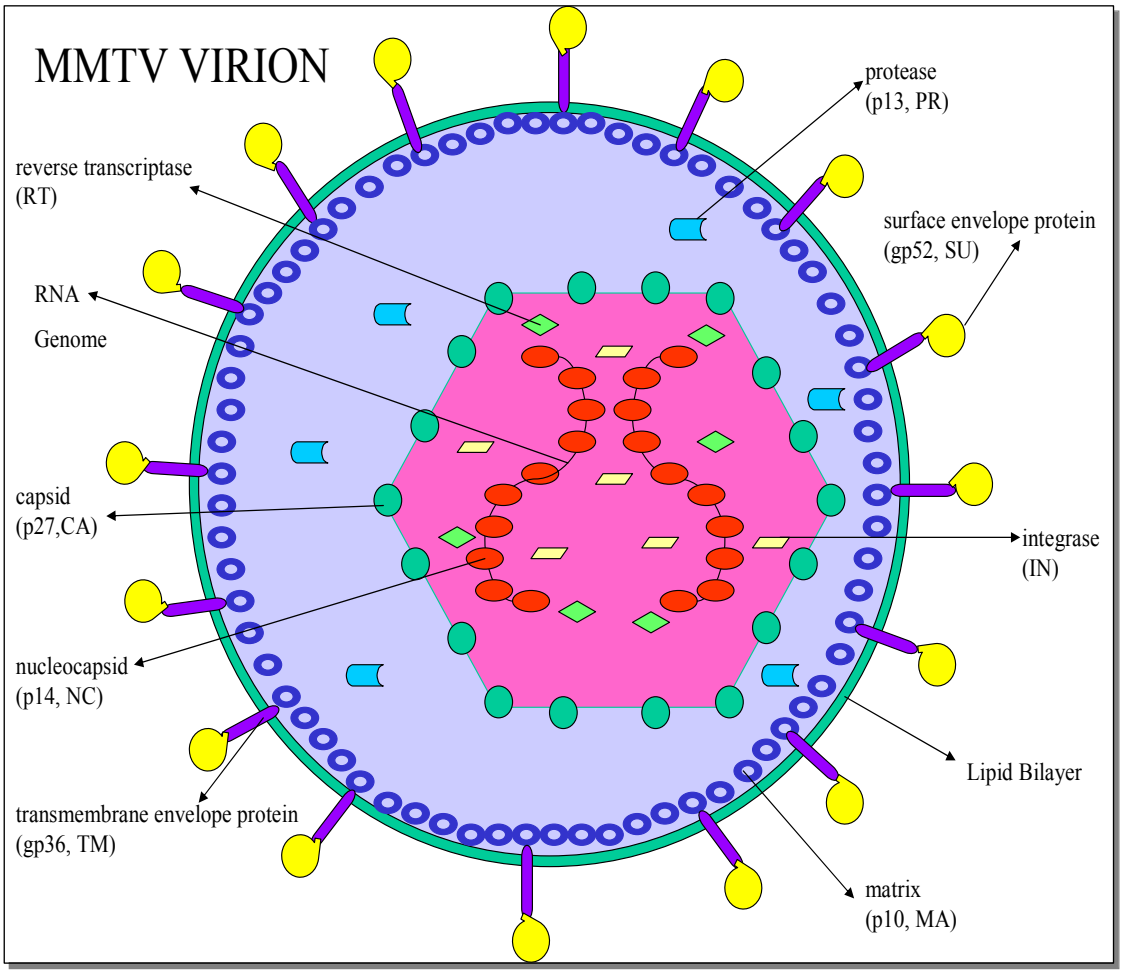


Figure 1. Structure of MMTV

## Introduction to the replication cycle of MMTV

MMTV has a replication cycle that is typical of retroviruses; though, it does have several features that are unique to its particular life history. A brief summary of the process begins when the viral surface glycoprotein, gp 52 binds to the transferrin receptor on the cell surface[16]. The virus enters the cell via clathrin-dependent receptor - mediated endocytosis and is therefore, enclosed in an endosome. The lower pH of the endosome causes membrane fusion to occur between the viral envelope and the host endosome membrane which delivers the capsid to the cytoplasm[17]. The virus then uncoats and viral RNA is reverse transcribed into proviral DNA in the host cytoplasm. The proviral DNA inserts randomly into the host genome[18]. Once proviral integration has occurred viral mRNAs are transcribed and the mRNAs are then translated into precursor Gag polyproteins in the cytoplasm[19]. The Gag polyproteins assemble into immature A particles in the cytoplasm with the genomic viral RNA in the core of the structure[20]. Viral Env proteins contain a signal sequence that directs their translation and subsequent processing to the endoplasmic reticulum. During maturation, the Env polyprotein is cleaved by a host protease to produce a surface glycoprotein and a transmembrane protein[21]. These cleaved proteins are then transported in vesicles to the cell membrane. As the immature A particles bud from the cell surface they acquire a lipid envelope that contains the viral surface glycoprotein and the viral transmembrane protein[22]. The immature A particles bud from the tips of long filamentous actin projections as mature virions[23] (Figure 2). A detailed review of the replication cycle of MMTV is presented in the following section of this paper.

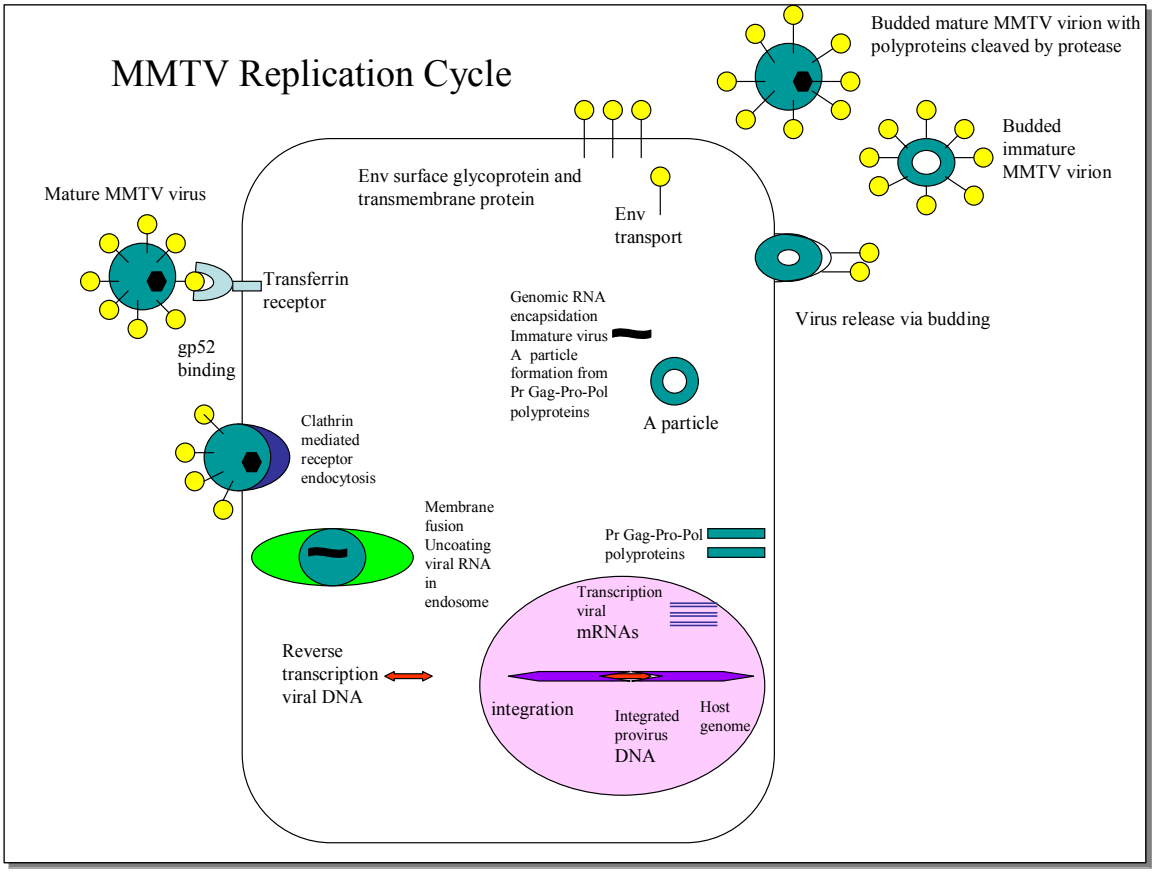


Figure 2. Replication cycle of MMTV

Replication cycle of MMTV

**Adsorption and Fusion**

The SU viral protein on the envelope of a mature virus interacts with the cell surface protein, transferrin receptor 1[17]. Transferrin receptor 1 is a single pass transmembrane glycoprotein with a short cytoplasmic tail that acts as a receptor for the virus. MMTV bound to the transferrin receptor 1 enters the cell via endocytosis of clathrin-coated pits. The vesicles containing MMTV traffic to acidic endosomes where virus-cell membrane fusion occurs [17]. The transferrin receptor 1 for MMTV is ubiquitously expressed in mouse cells and the gene for it resides on the mouse chromosome 16 [16]; still, MMTV transforms only mammary epithelial tissue and lymphoid tissue to produce tumors. The lack of tumor formation in other cells may be due to a transcriptional block in resistant cells [24].

**Reverse Transcription**

Non-coding sequences at the 5' end of the viral RNA genome contain the R or repeat sequence, the U5 region or 5' unique sequence, the PBS or the primer-binding region and the leader sequence. The R region is 15 bases long and it is repeated at both ends of the genomic RNA. The U5 region is important during reverse transcription allowing for the transfer of newly formed DNA from one end of the viral genome to the other end during the replication process. PBS, the primer binding sequence is complementary to the 3' nucleotides of the host tRNA[25]. The host tRNA, lys-3 acts as the DNA primer for the reverse transcriptase to synthesize minus-strand proviral DNA. The initial product of reverse transcription is an RNA-DNA complex. The RNase H

digests the RNA strand, which allows the reverse transcription of the viral genome into proviral DNA to proceed[26]. During reverse transcription of the viral genome into proviral DNA, the U5 region becomes positioned at the 3' end of the provirus[27]. The leader region of the viral genome contains the donor site for the generation of all spliced subgenomic mRNAs made by the virus and,Ψ, the packaging signal responsible for inserting the genomic RNA into the viral particles[28].

The non-coding sequences at the 3' end of the viral genome contain the polypurine tract that is made up of nine adenine and guanine nucleotides. This tract contains the initiation site for the synthesis of the + strand of viral DNA during the replication [29]. The unique sequence at the 3'end, U3, contains a sequence of ATT that is recognized by the integrase during the insertion of the provirus into the host genome after replication of the virus. After reverse transcription of the viral genome into proviral DNA, the U3 region becomes positioned at the 5' end of the provirus. U3 contains the transcriptional signals that the cellular machinery interacts with to initiate transcription of viral proteins. Lastly, the second R or redundant region has the poly A addition signal [30]. The reverse transcription of the viral RNA genome in the cytoplasm produces the proviral double-stranded DNA. Duplications of the U5 and the U3 regions create the two long terminal repeats, LTRs, found at both ends of the proviral DNA[31]. The coding sequences of the viral RNA genome and the proviral DNA of MMTV are *gag, pro, pol, env* and *sag* in this order from the 5' end of the genome [32].

**Integration**

Once the MMTV RNA genome has been reverse transcribed into linear proviral DNA the viral integrase randomly inserts the provirus into the host genome [33]. The provirus loses two base pairs, a cytosine and an adenine, from each end during integration. The host integration site consisting of six base pairs is duplicated on either side of the inserted MMTV provirus[34].

Although integration may be random, tumors from mice infected with MMTV show preferential proviral integration sites within four cellular gene families. These gene families are: the *Wnt* family; *Wnt-1*[35], *Wnt-3* [36] *Wnt-10b*[37], the fibroblast growth factor family; *Fgf-3/int-2*[38], *Fgf-4/hst*[39], *Fgf-8/AIGF*[40], *Fgf10*[41], the *Notch* family; *Notch-4/int-3*[42], and the *int-6* the gene encoding the p48 subunit of the eukaryotic translation initiation factor eIF3[43].

The *Wnt* gene family includes a group of 19 genes that encode glycosylated proteins that bind to cell signaling receptors that are important for mouse embryogenesis[44]. These proteins have an N- terminal hydrophobic signal peptide and 23-24 cysteine residues with highly conserved spacing, which may be important in the formation of disulfide bonds for protein folding [45]. It should be noted that *Wnt* genes are not normally expressed in the adult mammary gland [46].

The first *Wnt* gene *int-1* was discovered in 1982 in the genome of a mouse mammary tumor from an MMTV infected mouse [35]. It turned out to be a homolog to the Drosophila segment polarity gene, *wg,* wingless and the name was changed from *int-1*

*to Wnt-1*. [47] *Wnt-1* functions in the development of the mouse embryonic midbrain [48]. In 1987 the *Wnt*-3 gene was noted in the chromosome of a mammary tumor of an MMTV infected mouse and found to be a homolog to *Wnt-1* [36]. The analysis of DNA derived from a tumor of an MMTV infected mouse uncovered a gene, *Wnt 10b* that was homologous to the genes of the *Wnt* gene family [49].

The wingless signaling pathway is initiated when the secreted Wnt glycoprotein binds to Frizzled, a cell surface receptor. [50].Activation of the Frizzled receptor leads to the phosphorylation of Dishevelled a signaling protein. Dishevelled then inhibits a serine threonine kinase, GSK-3. [51] The inhibition of glycogen synthase kinase-3, GSK-3, allows β-catenin protein to be stabilized in the cytoplasm [52]. β-catenin accumulates in the cytoplasm and enters the nucleus. Once the β-catenin is in the nucleus it binds to the DNA binding proteins TCF/LEF. TCF, T-cell factor, and LEF, lymphoid enhancer factor, are transcriptional activators of cellular genes. The inappropriate activation of the Wnt pathway leads to the activation of gene transcription leading to cell growth and proliferation [53].

The fibroblast growth factor, *Fgf*, gene family consists of 21 genes that code for small peptides. These molecules are secreted from the cell as peptide growth factors. They act as ligands for receptor tyrosine kinases, RTKs. The *Fgf* genes 3, 4, and 8 are expressed in the primitive streak stage mouse embryos and are necessary for signaling in gastrulation , limb, and CNS development [54]. Activation of the *Fgf* genes is due to the insertion of the MMTV provirus next to the proto-oncogene. The viral enhancer located in the 5' LTR affects the transcriptional promoter of the adjacent *Fgf* gene [38].

In 1984, analysis of the genome of a tumor located in a mouse infected with MMTV located the MMTV provirus inserted next to the gene for *Fgf3/int-2*[55] [56]. Then in 1989, the MMTV provirus was found integrated near the *Fgf 4/hst* gene in the chromosome of a tumor of an MMTV infected mouse [39]. In addition, analysis of the DNA from a tumor from an MMTV infected mouse isolated an insertion of the provirus near the *Fgf8* gene [57].

The fibroblast growth factor pathway involves the fibroblast growth factor as a ligand that activates a receptor tyrosine kinase. The receptor tyrosine kinase can activate multiple cell signaling pathways resulting in kinase cascades and the activation of cellular transcription factors for cell growth and proliferation [58].

The *int-6* MMTV integration site was noted in mammary tumors of Czech II mice in 1995[59]. Then in 1997 the gene for *int-6* was found to be the gene for eIF3-p48, the eukaryotic translation initiation factor 3 p48 subunit[43]. The eIF3-p48 is involved in the dissociation of the 80s ribosomal complex, the association of the eIF2-GTP-Met-tRNA and the binding of eIF5 to the mRNA to begin translation. Increased levels of cyclin D protein has been noted when eIF4E was over expressed in NIH3T3 cells [60] and in MAC-T cells [61]. Increased levels of cyclin D lead to the growth and proliferation of mammary gland cells[61].

Another MMTV proviral integration site that was present in mouse mammary tumors of Czech II mice is *Notch4/int-3*. The *Notch4/int-3* gene encodes a transmembrane receptor protein that is a member of the Notch receptor family. Notch

receptor proteins function during development for cell fate decisions. Notch proteins activate the transcription factors CBF1, Supressor of Hairless, and LAG-1[42]. The MMTV proviral integration in mammary gland cells leads to the expression of a truncated *Notch4/int-3* gene. The translated Notch4/int-3 protein is the intracellular part of the Notch receptor protein. The truncated Notch receptor is constitutively active and this leads to the growth and proliferation of mammary gland cells [62].

The co-operativity of oncogenes has been shown to result in faster growing tumors in MMTV infected transgenic mice. The transgenic mouse strain MMTV-*Wnt1* subsequently infected with MMTV has produced tumors that have proviral integrations next to the *Fgf3/int-2*, *Fgf4/hst,* or *Fgf8* genes [40] [63]. Accelerated tumor growth also occurs in MMTV-*Fgf3* transgenic mice that have a MMTV proviral insertion next to the *Wnt-1*or *Wnt-10b* gene [37].

Inbred strains of mice such as; A, DBA, C3H, GR, BR6 and RIII have been used to study mammary tumorigenesis in mice because these mice developed mammary tumors within a short period of time [64]. The C3H and GR inbred mouse strains have an integrated endogenous MMTV proviral genome, Mtv-1 or Mtv-2 that is transmitted vertically in the germinal cells. In some of the inbred mouse strains the endogenous MMTV encodes an infectious virus. These mice develop pregnancy independent tumors in the first two years of life [65] [66]. When these inbred mouse strains were studied, it was found that different strains had a different frequency of MMTV insertions into the *Wnt* genes, the *Fgf* genes, the *Notch* genes, and the *eIF3p48* gene [67] [68]. This suggested that inbreeding of these inbred mouse strains fixed MMTV insertional proviral

mutations in the germ cells. These insertional mutations allowed a greater incidence of MMTV infection and subsequent tumorigenisis in the mammary cells of these mice [69].

Recently, studies have identified a full-length human endogenous retrovirus (HERV- K) with sequence homology to MMTV [70]. In addition, a 660-bp sequence, which, is 96% homologous to the MMTV env gene sequence but only 18% homologous to (HERV-K), has been found in human breast tumors; however, at present it is not known whether these integrated MMTV-like *env* sequences in the human genome are of endogenous or exogenous origin[71] [71, 72] .

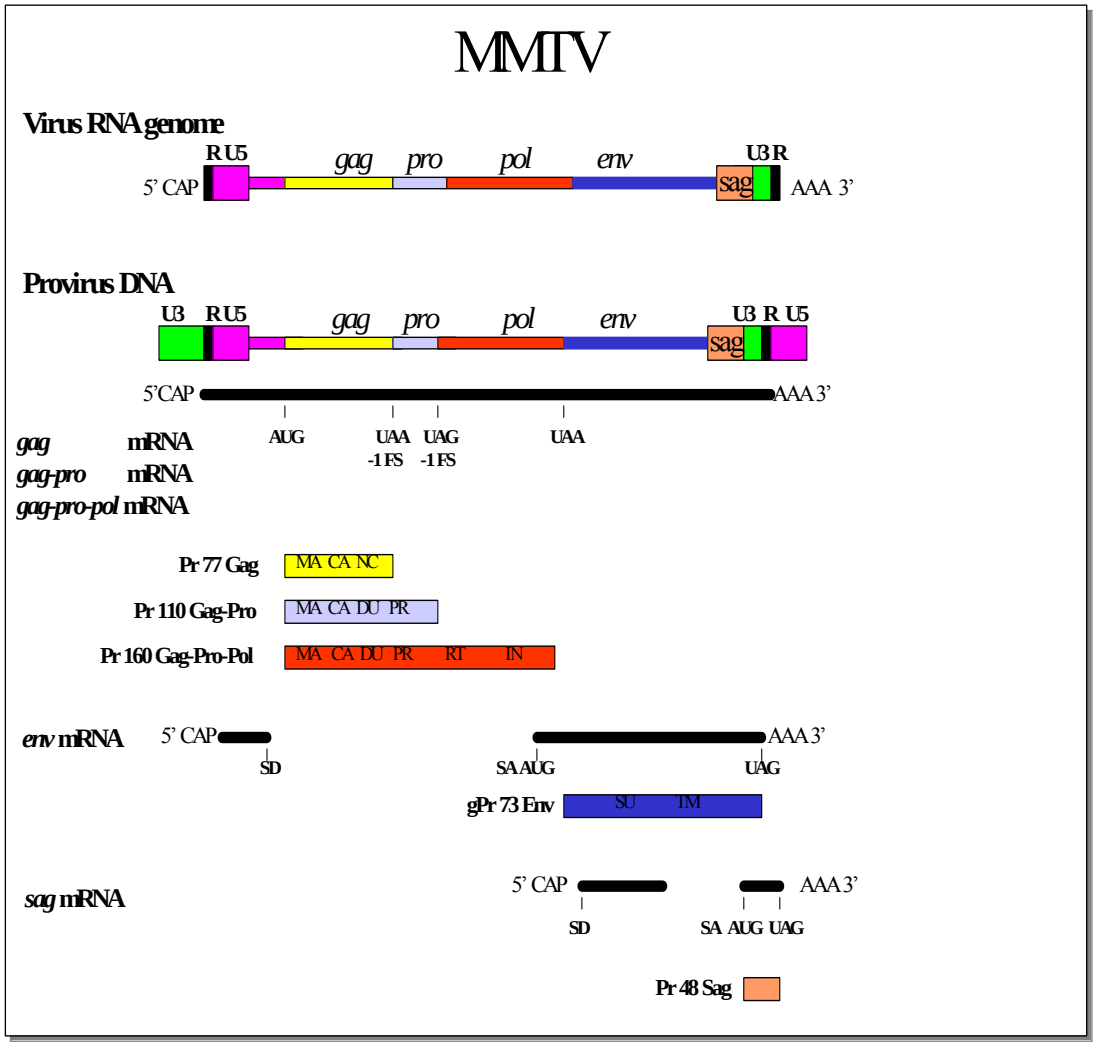


Figure 3. MMTV RNA genome

MMTV proviral DNA, a product of reverse transcription

MMTV mRNAs, products of transcription using host cellular machinery

MMTV precursor proteins, products of translation using host cellular machinery.

**Transcription**

The integrated MMTV LTR is a stretch of DNA (1195 base pairs in length) at the 5' end of the viral genome that is responsible for the expression of the virus in mammary epithelial cells and for the activation of cellular proto-oncogenes. For example, insertion of MMTV next to genes of the *Wnt* gene family are believed to be responsible for the tumorigenesis of virally induced mammary adenocarcinomas in transcriptionally permissive cells [73]. Within the LTR there is a weak promoter made up of a NF-1 binding sequence, three overlapping octamer motifs, a TATA element, and a biding site for TFIID, which induces basal transcription of the virus[74]. The MMTV promoter is also a steroid hormone-inducible promoter that has multiple hormone response elements, HREs, located in a region -200 to -80 upstream from the transcription initiation site. The glucocorticoid receptors (GR) bind to the HREs and stimulate increased production of virus in mammary cells [75].

MMTV also infects the epithelial cells of the salivary gland, lungs, kidney, prostate gland, seminal vesicle, testes and lymphoid tissue. These cells also have glucocorticoid receptors[76]; nonetheless, the expression of virus in lactating mammary epithelial cells is five hundred times greater than in other types of infected epithelial cells[77]. In the late 1980's, mammary cell specific transcription factors were identified[78, 79]. These mammary cell-specific factors bind to enhancer regions on the proviral DNA and interact with the gluococorticoid-inducible promoter to produce large numbers of virions in mammary cells[80].

There are two regions of the LTR, which have been designated as mammary cell-specific enhancers, the Ban2 enhancer and the MEM element. The Ban2 enhancer, -1075 to -978 upstream from the transcription start site has sequences that bind the transcription factors AP-2, a member of the CTF/NF-1 (nuclear factor 1) family, MAF, an Ets -related mammary cell activating factor and mp4, which has not been identified [81] [82] [83]. The MEM element, located -956 to -862 with respect to the proviral transcription start site, has three binding sites for nuclear proteins[31]. These proteins have been identified as MAF, NF-1, and F4[84]. F4 is a protein that is found only in mammary cells. This MEM element can enhance the MMTV proviral promoter or the promoter of a cellular proto-oncogene specifically in mammary cells; yet, the binding of only the mammary-specific protein, F4, to the enhancer is not enough to enhance a promoter. Therefore, the combinatorial binding of all of the nuclear factors to the enhancers is necessary for mammary cell-specific transcription of proviral MMTV or cellular proto-oncogene [85] [86].

The LTR has two negative response elements, NREs. The distal dNRE is -638 to -455 bps upstream of the transcription start site and the proximal pNRE is -364 to -263 with respect to the transcription initiation site. The protein SATB1, special AT-rich binding protein, binds to the NRE to repress transcription of MMTV in tissues such as the thymus [87]. Another transcriptional repressor that binds to the pNRE of the MMTV LTR is the CAAAT displacement protein, CDP [88]. This repressor protein is downregulated during the development of the mammary gland. As the mammary gland matures and the cellular RNA polymerase II transcribes the MMTV provirus, MMTV

virions are produced [89]. Transcription of the provirus produces full- length mRNA, spliced mRNAs, as well as progeny RNA. Full-length mRNAs are the *gag* mRNAs, *gag-pro* mRNAs, and *gag-pro-pol* mRNAs[90] [91]. The spliced mRNAs are the *env* mRNAs and the *sag* mRNAs[92] [93].

**Translation**

The viral mRNAs are translated into the viral proteins in the cytoplasm using the host cellular machinery. The *gag* mRNA is translated into the precursor protein Pr77, a Gag polyprotein. Proteolytic processing of Pr 77 by the viral protease produces the structural proteins: the matrix protein p10 or MA; the capsid protein p27 or CA; the nucleocapsid protein p14 or NC, and some smaller proteins p21, p3, and p8 that have no known function[32, 94]; though, p21 is cleaved into a few smaller proteins [90].

The matrix protein of the virion interacts with the plasma membrane of the cell. The amino terminus of the protein is myristylated at the first glycine. This myristylation allows the matrix protein to interact with the lipid membranes [95]. Capsid protein forms the structural shell of the core of the virion. It is thought to enclose the ribonucleoprotein complex, which contains the genomic RNA. The capsid protein appears to be important in the assembly of the immature capsid [96]. The nucleocapsid protein has a sequence that includes many basic amino acids and a cysteine-histidine box that is similar to a zinc finger domain seen in DNA-binding proteins [97]. This zinc finger domain likely explains why the nucleocapsid encapsulates the viral RNA in the cytoplasm [98].

Several different polyproteins are generated from one viral RNA by frameshifts during translation. The *gag-pro* mRNA is translated as a precursor protein Pr 110, a Gag-Pro polyprotein. This protein is created by a -1 frameshift at base 2082[99]. Within Pr 110, the NC sequence of the *gag* gene and the first part of the *pro* gene comprise a *dut* gene, which encodes p30 or DU a dUTPase. This enzyme destroys any deoxyuridine so that it cannot be incorporated into the proviral DNA [100] [101]. The C terminus end of the *pro* gene codes for p13 or PR the viral protease [102]. Two -1 frameshifts located at bases 2082 and 2891 during the translation of the viral mRNA encode a large precursor polyprotein. The precursor protein Pr160, a Gag-Pro-Pol polyprotein encodes a viral reverse transcriptase with RNase H activity and an integrase [103].

The reverse transcriptase is a DNA polymerase that needs a RNA primer to initiate DNA synthesis. A host tRNA lys-3, that is incorporated into the virion acts as the primer for DNA synthesis of the first minus DNA strand of the provirus[26]. The polyproline tract of the viral RNA genome acts as the primer for the creation of the plus DNA strand of the provirus[15]. The polyprotein Pr160 also has the viral integrase in its C terminus. The integrase contains a cysteine-histidine “zinc finger” DNA binding motif. This allows the enzyme to bind to the proviral long-term repeats, LTRs and then integrate the proviral DNA into the host genome [104].

Transcription of the proviral DNA also produces a separate spliced *env* mRNA that has a splice donor site at base 288 and a splice acceptor site at base 5363. The *env* mRNA is translated into a precursor protein, Pr 73 Env that contains a signal sequence targeting it to the endoplasmic reticulum. Env is N-glycosylated in the ER and then

moves to the Golgi where a cellular protease cleaves the Env poly protein to produce gp52, SU a viral surface envelope protein and gp36, TM a viral transmembrane envelope protein. SU and TM remain attached to each other via disulfide bonds [21]. They travel to the host plasma membrane in vesicles and are then incorporated into the lipid envelope of the mature B type virion when the immature A particle buds from the host cell[22].

The third distinct mRNA is generated from transcription of the *sag* gene located in the U3 region of the LTR. The sag RNA must be spliced and contains a splice donor site at base 6143 and a splice acceptor site at base 7305. The *sag* mRNA is translated into a precursor protein Pr 48, SAg, a superantigen protein. The Pr 48 is modified in the host endoplasmic reticulum with five N-linked glycosylation sites. A cellular protease in the Golgi cleaves Pr48 and produces SAg protein [105]. The Sag protein is an integral membrane protein with a large glycosylated extracellular domain that moves in vesicles to the plasma membrane of the cell. The expression of Sag with its glycosylated extracellular domain on the class II major histocompatibility complex proteins is located on the cell membrane of B-lymphocytes. Sag is essential activation of certain T cell subsets containing Vβ TcR [106] (Figure 3).

**Assembly**

MMTV utilizes a polyprotein strategy for viral assembly. This process insures that none of the individual proteins; matrix, capsid, and nucleocapsid, are individually targeted to the membrane. The order of the proteins in the polyproteins corresponds to their relative location in the virion from the outside of the virus to the inside of the virus. MMTV polyproteins assemble into a capsid shell or immature A particle in the

cytoplasm. The formation of the A particle occurs prior to its transport to the plasma membrane [22]. The viral envelope proteins are transported to the plasma membrane via the host secretory pathway. The A particles associate with the cell membrane. They acquire a viral lipid envelope with viral envelope proteins as the virus buds from the host cell [107] [21].

**Budding**

The budding process of MMTV seems to involve the host actin cytoskeleton. The immature viral A particles have been observed at the tips of long filamentous projections [23]. In addition, cytochalasin D, which disrupts the actin cytoskeleton, reduced the number of mature viral B particles released into the supernatant by 80% [108]. Thus budding of MMTV appears to be an actin-dependent process.

Insight into how MMTV interacts with the actin cytoskeleton when it is released from infected cells is very incomplete. We utilized "modulated polarization microscopy" to monitor the components of the cytoskeleton in living cells without endogenous or exogenous labels [109]. Cells infected with MMTV initiated the formation of filamentous projections with virus particles at their tips. These filopodial projections emerged from the cell surface of infected live cells when viral production was hormonally induced with dexamethasone. The virions were observed to shoot out of the ends of the filopodia. This led us to investigate the relationship of MMTV with the actin cytoskeleton during viral egress from the cell.

# Chapter 2: Cdc42 signal transduction pathway

Characterization and regulation of the Rho small G-proteins

Rho GTPases are members of the Ras superfamily of small monomeric G-proteins. At present, nineteen mammalian Rho family small GTPases have been isolated: Rho (A B C isoforms), Rac (1, 2, 3 isoforms), Cdc42 (Cdc42Hs, G25K isoforms), Rnd1, Rnd2, RhoE/Rnd 3, RhoH/TTF, RhoG, CHP, WRCH1, Rho D, RIF, TCL, and TC10 [110]. The most completely characterized members of the Rho family are Rho, Rac, and Cdc42. The structure of the Rho family proteins is very similar. The feature that distinguishes Rho family members from the rest of the Ras family is a sequence of 13 amino acids termed the insert region that forms an extra α- helical domain that is not present in Ras[111-114].

G proteins exist in two conformations depending on whether they are bound to GDP or GTP. The key conformational changes occur in the switch I and the switch II regions of the protein [115]. The switch regions overlap the effector region such that GTP binding controls whether the G protein will interact with an effector or not [116]. The C-terminus of the Rho GTPases contains a CAAX box that specifies post-translational prenylation. This processing consists of the addition of a 20-carbon geranylgeranyl group to the terminal cysteine SH followed by cleavage of the last three amino acids and methylation of the terminal carboxyl group. The prenylation allows the GTPases to interact with lipid membranes [117] .

The Rho small GTPases act as molecular switches. They cycle between an active GTP-bound form and an inactive GDP-bound state. In the active GTP-bound form of Rho the amino acid threonine 35 in the switch I region and glycine 60 in the switch II region bind to the γ-phosphate of the GTP. When GTP hydrolysis occurs, the interactions between threonine 35 and glycine 60 terminate and result in the quiescent GDP-bound form of Rho and a free phosphate [113].

Regulation of the cycling of the Rho family GTPases is accomplished through the binding of guanine nucleotide exchange factor proteins (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitor proteins (GDIs)[118-120] (Figure 4).

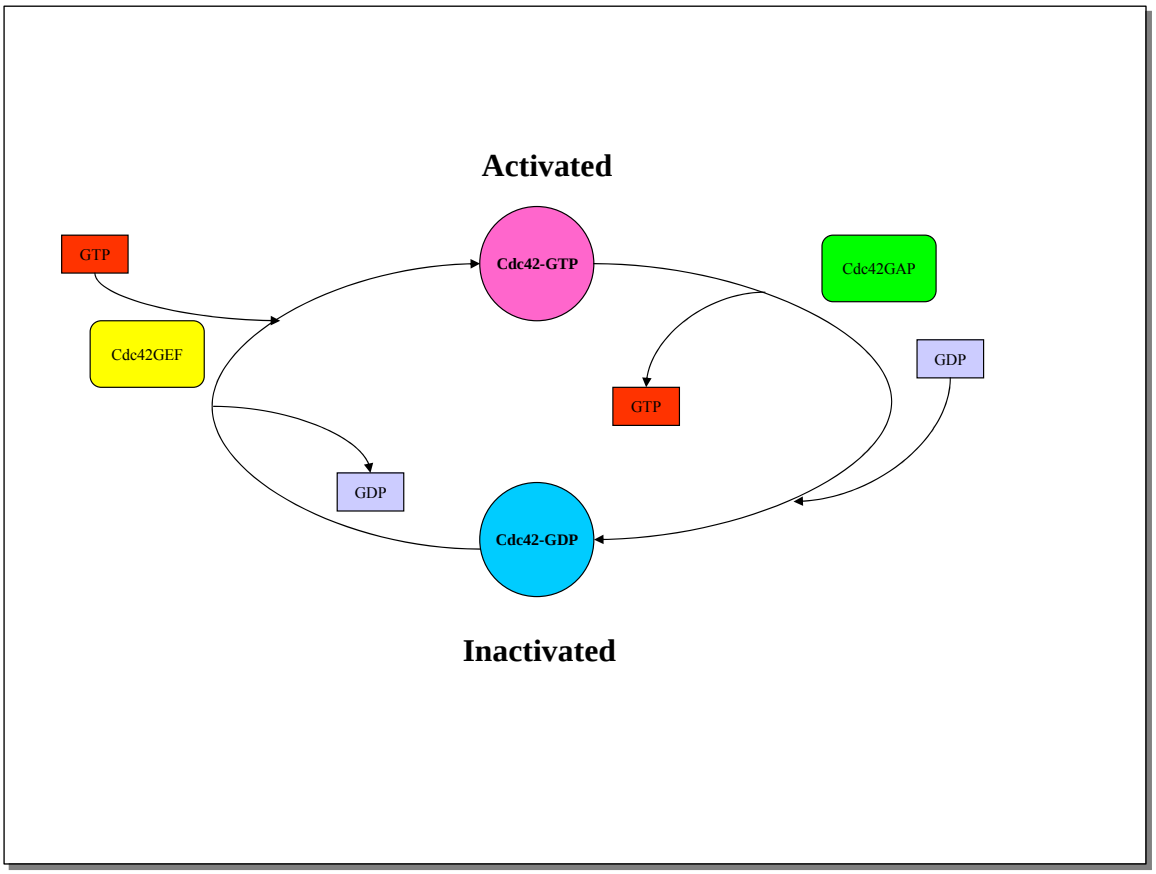


Figure 4. Regulation of Rho GTPases

Activation of cell surface growth factor receptors and integrins stimulate particular GEFs that then activate specific Rho small GTPases. GEFs have an exchange factor domain with catalytic activity termed the Dbl homology domain (DH). This domain was first isolated as the *Dbl* oncogene [121]. Deletion analysis of the Dbl protein showed that the Dbl homology domain was necessary and sufficient for activation of Rho family GTPases[122]. The C-terminal end of the DH domain is connected to a pleckstrin homology domain (PH). The pleckstrin homology domain has been shown to bind phospholipids. Phospholipid binding leads to the localization of the GEFs at the membrane where activation of the Rho small GTPases occurs [123].

Recently, a new family of GEFs , the DOCK 180 superfamily, has been identified[124]. These novel GEFs have been shown to activate Rac and Cdc42 [125] [126]. The exchange factor domain of these Dock 180 proteins known as; the Docker domain [125], CZH2 domain [126] or the DHR-2 domain [124] respectively, is not homologous to the Dbl homology domain of Dbl GEF proteins. These novel Dock 180 GEFs do not have pleckstrin homology domains and require the additional binding partners known as ELMO and Crk to function as GEFs[127]. Another Dock 180 protein known as Dock9/zizimin 1 dimerizes before it activates Cdc42[126].

Inactivation of the Rho family small GTPases occurs through GTP hydrolysis. Intrinsic GTP hydrolysis by Rho family proteins is very slow but can be accelerated several orders of magnitude by interaction with GTPase-activating proteins (GAPs). These proteins have a GAP domain which is homologous to the C-terminal domain of

*Bcr,* the break-point cluster region gene product. Once GTP is hydrolyzed and converted to GDP the G protein can no longer interact with effector molecules and the signal terminates [119].

Rho proteins can exist in a complex with guanine nucleotide dissociation inhibitor proteins, Rho GDIs, in the cytoplasm. RhoGDI proteins bind to the prenylated CAAX sequence at the carbonyl terminus of the Rho GTPase. The prenylated C terminus of the RhoGTPase is inserted into a hydrophobic pocket in the RhoGDI[128]. When RhoGDI binds to the CAAX box it causes release of Rho from the membrane which is normally where it is active. Three Rho GDIs have been identified in mammals; Rho GDI-1, D4/LyGDI, and GDIγ/ RhoGDI-3 [129, 130]. Generally, GDIs prevent nucleotide exchange or GEF activation of the small GTPases; alternatively, GDIs prevent intrinsic or GTPase-activating proteins (GAPs) from hydrolyzing GTP for inactivation of the small GTPases [131].

Cdc42 and downstream effectors

Cdc42 was originally isolated in the yeast *Saccharomyces cervisiae* as a cell cycle mutant that caused defects in budding and cell polarity [132]. Then the mammalian isoforms, G25K and Cdc42H were found to be two different spliced variants of the same gene [133] [134].

The structure of mammalian Cdc42 in both the GDP-bound form and the $GppCH_2P$-bound form was determined with NMR. The only major difference from other Rho family proteins was that the conformational change of Cdc42 in the GTP-bound state included not only the switch I loop ( residues 26-45) and the switch II loop (residues 59-74) but also the helix $\alpha 3$ and the $\alpha 3$-$\beta 4$ loop. This GTP dependent conformational change of the effector region creates a larger switch area to interact with downstream effectors [112].

Downstream effectors of Cdc42 were identified using the yeast two-hybrid system and affinity chromatography[135]. The potential effector proteins for Cdc42 are listed in Table 1. These effector proteins interact with the GTP bound form of Cdc42. The main difference in conformation between the GTP-bound form and the GDP-bound form of Cdc42 is in the switch I and switch II loops[115]. Thus, the effector proteins must be able to discern between the GTP-bound form and the GDP-bound form in order to interact with the GTP-bound conformation of the switch I region[116]. In addition, some effector proteins also interact in a GTP-dependent manner with different regions of Cdc42 outside

of the switch I region, such as the Rho family α-helical insert region or the C-terminal region[136].

| Potential Effector Protein | Type of Protein | Functions | Binding Motif |
|---|---|---|---|
| WASP | scaffold | actin organization | CRIB |
| MSE55, BORGs | scaffold | actin organization | CRIB |
| Pak 1,2,3 | Ser/Thr kinase | actin organization/JNK | CRIB |
| Pak 4 | Ser/Thr kinase | actin | CRIB |
| MEKK 1,4 | Ser/Thr kinase | JNK | CRIB |
| Mlk 2,3 | Ser/Thr kinase | JNK | CRIB |
| MRCK $\alpha$, $\beta$ | Ser/Thr kinase | actin | CRIB |
| ACK 1,2 | non-receptor Tyr kinase | endocytosis | CRIB |
| Par 3,6 | polarity protein | tight junction maintenance | ? |
| p70 S6 kinase | Ser/Thr kinase | RNA processing | ? |
| COP 1 | coatomer complex | vesicular transport | ? |
| IQGAP 1,2 | scaffold | actin/ cell-cell | ? |
| CIP 4 | scaffold | actin organization | ? |
| PI3 kinase | Lipid kinase | PIP3 levels | ? |
| PLD | Lipase | Phosphatidic acid levels | ? |
| PLC_$\beta$2 | Lipase | DAG/IP3 levels | ? |
| p85 Cool-1/$\beta$-PIX | p21-activated kinase-binding protein | EGF receptor regulation | ? |

Table 1. Potential effectors of Cdc42 and their biological functions and binding motifs

Recently NMR has been used to determine the structures of Cdc42 bound to WASP, the Wiskott-Aldrich Syndrome Protein and to ACK, Activated Cdc42-associated tyrosine Kinase. These studies have shown that amino acid Asp 38 in switch I is essential for selective binding of Cdc42 to the Cdc42-Rac interactive binding domain, CRIB, domains of ACK and WASP[137] [138]. Mutations substituting Glu for Asp 38 in Cdc42 have shown that there is decreased binding affinity of Cdc42 for the CRIB domain of PAK 3, p21-activated kinase [139].

Generally, Cdc42 activation of effector proteins occurs with the release of intra-molecular auto-inhibitory interactions and the subsequent activation of the functional domains within the effector molecules. A scaffold effector molecule such as WASP/N-WASP exists in an auto-inhibited conformation with an N-terminal regulatory domain that blocks the activity of the C-terminal activation domain [140]. Similarly, the serine/threonine kinases PAK 1-3, have intra-molecular regulatory domains that inhibit the catalytic domain [141]. This inhibition is relieved when Cdc42 binds and prevents binding between the auto-inhibitory region and the catalytic domain.

Cdc42 regulates biological responses in the cell

The biochemical and cellular functions of Cdc42 have been clarified using dominant-negative and constitutively active mutants (Figure 5). Mutagenesis studies identified a mutant containing an amino acid substitution of an asparagine for a threonine at position 17 that suppressed the function of Cdc42 resulting in a dominant negative mutant. This mutant functions as a dominant negative Cdc42 by competing for Cdc42GEFs. When it was expressed in cells it binds to the GEFs and forms unreactive complexes that are unable to produce a downstream response [142]. Alternatively, Cdc42 mutants that involved the substitution of leucine for glutamine at amino acid 61 or the substitution of valine for glycine at residue 12 prevented both the intrinsic Cdc42 GTPase activity and GAP-induced hydolysis of GTP[143]. This results in a constitutively active Cdc42.

Cdc42 has multiple downstream effectors. Some of the effectors transmitt their signals through kinase cascades to obtain a biological response while, other effector pathways still need to be clarified. One of the main functions of Cdc42 is in linking receptor signals to reorganization of the actin cytoskeleton [144]. In addition, Cdc42 is linked to other biochemical pathways such as vesicular transport, endocytosis, the cell cycle, activation of mitogen-activated protein kinase cascades, regulation of transcription factors, and cellular transformation [145].

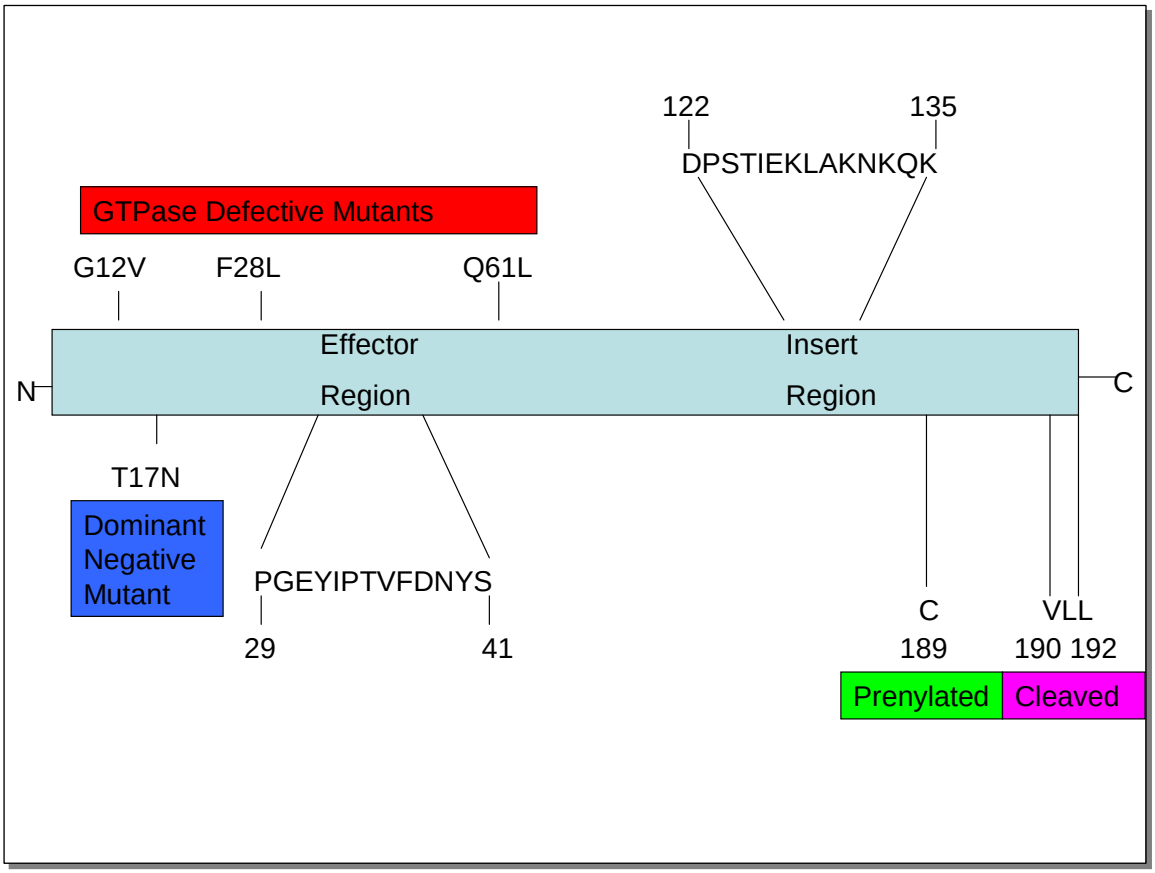


Figure 5. Structurally important regions of Cdc42

Cdc42 and the regulation of the actin cytoskeleton

One of the ways that Cdc42 affects the cell is through the formation of filopodia. Filopodia are thread-like or spike-like actin-containing structures that form at the cell surface. These filopodia have been implicated in sensory function in neural growth cones [146]. In addition, they have been linked to the budding process of MMTV [23] [147] [108].

Cdc42 mutants have been useful in elucidating the role of Cdc42 in formation of filopodia. For example, microinjection of constitutively active Cdc42, Cdc42(Q61L) and Cdc42(G12V), into mammalian cells resulted in the production of numerous filopodia; whereas, microinjection of the dominant negative mutant of Cdc42, Cdc42(T17N), blocked formation of filopodia. Since growth factors can stimulate formation of filopodia, additional studies were done to determine if Cdc42 functioned in a normal growth factor signaling pathway. A variety of growth factors were examined and bradykinin was found to specifically elicit the formation of filopodia. However, a dominant negative Cdc42(T17N) was able to block filopodia formation in the presence of bradykinin. These results indicated that bradykinin activates Cdc42 and leads to the formation of filopodia via a signal transduction pathway [142] [144].

WASP is another protein implicated as a Cdc42 effector. WASP was identified as a protein that was altered in patients with the Wiskott-Aldrich syndrome[148] and caused

defects in actin-dependent processes. For example, patients have reduced mobility of lymphoid immune cells and megakaryocytes that do not form filopodia [149]. It was found that the WASP protein is expressed in hemapoetic cells and its homologue N-WASP is expressed ubiquitously [150]. Microinjection of N-WASP and the expression of constitutively active Cdc42(G12V) produced filopodia; while, anti N-WASP prevented the formation of filopodia[151]. This suggested that the induction of filopodia formation was dependent on activated Cdc42 and N-WASP (Figure 6).

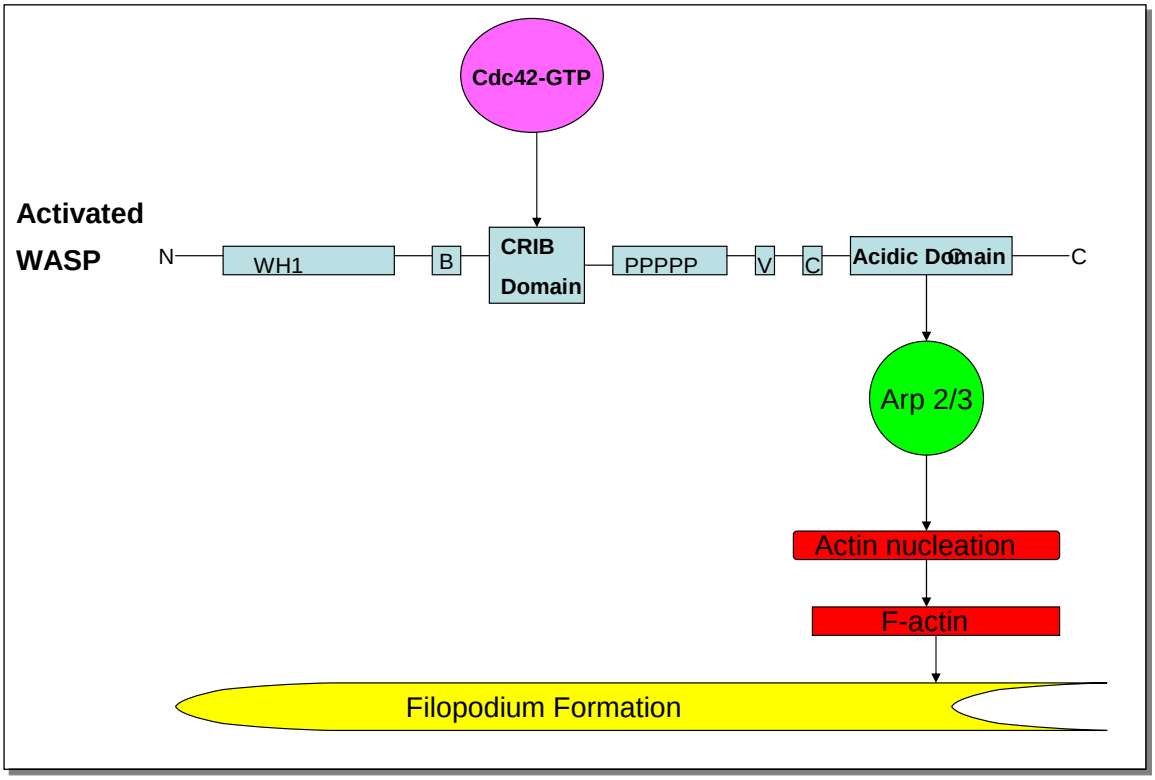


Figure 6. Cdc42/ WASP/Arp2/3 Pathway

Activated Cdc42-GTP binds to the Cdc42/Rac interactive binding, CRIB, domain on WASP. Activated WASP recruits actin-nucleating Arp2/3 complex to the to its C-terminal acidic domain. The Arp 2/3 complex nucleates the formation of actin filaments, F-actin. Activated Cdc42 is linked to actin polymerization and filopodia formation through the activation of WASP and stimulation of actin nucleation with the Arp2/3 complex.

All Wasp and N-WASP proteins have conserved sequence motifs. These motifs include a WASP homology domain 1 (WH1), a Cdc42/Rac GTPase binding domain (GBD), a proline-rich domain, a G-actin binding verprolin-homology domain (VH) or a WASP homology domain 2 (WH2), a cofilin-homology domain (CH) and a carboxyl-terminal acidic segment (A). WASP has a single WH2 domain while N-WASP has tandem WH2 domains [148].

The WH1 domain encompasses the first 150amino acids and in a recent biochemical study it was found to bind constitutively to WASP interacting protein (WIP). It is a proline-rich protein that has a verprolin homology domain that has a KLKK motif that binds to G-actin and F-actin. It is homologous to the proteins Cr16 and WICH [152]. WIP is a member of a family of regulators of WASP-mediated actin polymerization and it may act as a functional unit in filopodia formation [153]. In addition, WIP has been found bound to Wasp in actin-tails produced during *Shigella* and *Vaccinia* infection [154] [155].

The WASP B domain has been found to bind to profilin [156]; while, the WASPGBD domain binds to activated Cdc42. Binding of the activated Cdc42 activates WASP by releasing the auto-inhibition that occurs between the region C-terminal to the GBD and the carboxyl-terminal acidic region [140].

The proline-rich region of WASP binds to SH3 domains of adaptor proteins such as Nck and Src-type kinases. Nck may be involved in signal transduction pathways involving Wasp-mediated actin polymerization [157].

Activated WASP recruits G-actin to the VH domain and an actin-nucleating Arp2/3 complex to the to its C-terminal acidic domain [158]. The Arp 2/3 complex is able to nucleate the formation of actin filaments that grow from the barbed or (+) end [159]. Thus, Cdc42 is linked to actin polymerization and filopodia formation through the activation of WASP and stimulation of actin nucleation with the Arp2/3 complex (Figure 7).

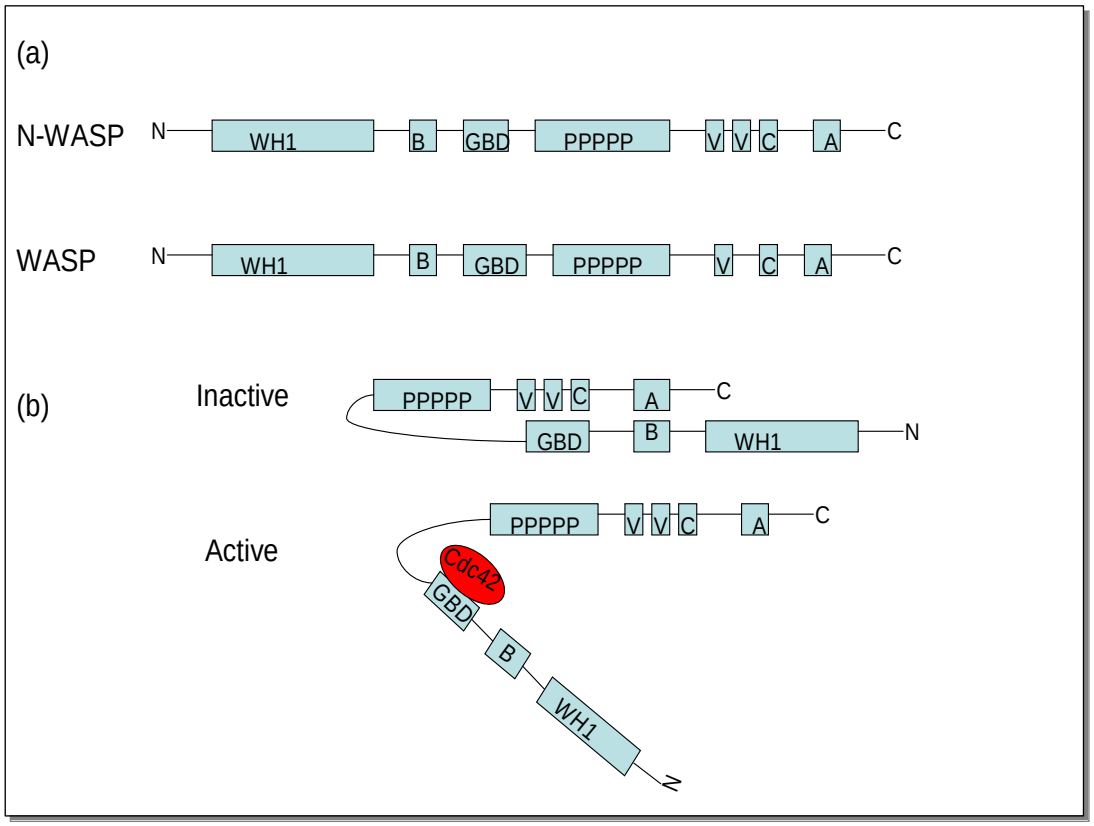


Figure 7. Domains of Wiskott-Aldrich Syndrome proteins WASP and N-WASP

(a) The WASP homology 1 domain (WH1) is conserved in WASP and N-WASP. The B domain is a series of basic amino acids which binds profilin.The GTPase binding domain (GBD) binds to activated Cdc42 or activated Rac. The polyproline domain (PPPPP) binds to SH3 domains of adaptor proteins. The verprolin homology domain (V) binds to G-actin and acts in concert with the cofilin homology domain (C) and the C-terminial acidic segment (A) to interact with the ARP2/3 complex for the initiation of actin polymerization. (b) The WASP and N-WASP proteins are in an inactive auto inhibited state. Activation occurs when activated Cdc42 binds to the GTPase binding domain (GBD) disrupting the auto inhibition.

Cdc42 regulation of gene transcription

In recent years, Cdc42 has been identified as a molecular switch that functions in signal transduction cascades to regulate the activity of transcription factors and gene transcription. Constitutively active Cdc42 is able to activate mitogen-activated protein kinase cascades. Activated Cdc42 activates the serum response factor (SRF) which complexes with the Elk-1 transcription factor at the serum response DNA element found in the promoter sequences of the *c-fos* gene[160]. Over expression of activated Cdc42 in mammalian cells leads to the activation of the c-Jun $NH_2$-terminal kinase (JNK) pathway [161] [162]. JNK activation can lead to the phosphorylation and activation of the transcription factors Jun and ATF-2 which complex at the Activator Protein-1, AP-1. AP-1 is a DNA element found in the promoter sequences of the *c-jun* gene. JNK activation can also lead to the activation of the Elk-1 transcription factor that binds the serum response DNA element of the *c-fos* gene [163, 164]. Over expressed activated Cdc42 has been shown to activate the p38 mitogen-activated protein kinase cascade. Phosphorylated p38 kinase activates the transcription factor ATF-2 which binds to the AP-1 DNA element of the *c-jun* gene [165].

Constitutively active Cdc42 activates the c-Jun N-Terminal kinase kinase kinase (MEKK1) signal cascade which results in the phosphorylation of IκB and the subsequent activation of the transcription factor NFκB and subsequently the NFκB responsive genes [166]( Figure 8).

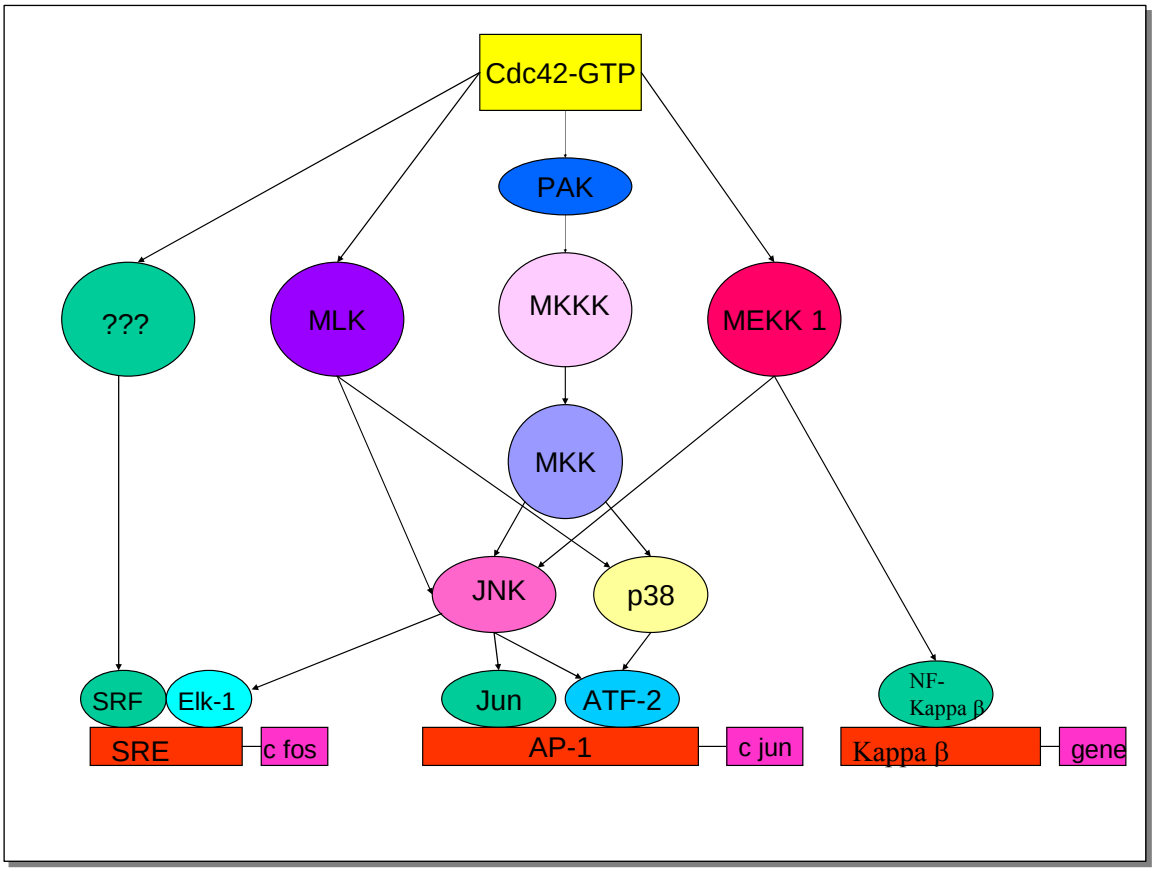


Figure 8. Cdc42 regulation of gene transcription

Cdc42 regulates cell proliferation

Cdc42 induces cell-cycle progression by up regulating the levels of cyclin D, cyclin E and cyclin A. One of the first studies showed that the injection of activated Cdc42 (G12V) into quiescent fibroblasts led to cell-cycle progression through G1 and to DNA synthesis. The injection of dominant negative Cdc42(T17N) into serum-stimulated fibroblasts blocked DNA synthesis [167].

Specific cyclins bind to and activate particular cyclin-dependent kinases (CDKs). The interaction of elevated levels of cyclin D with CDK4 and CDK6 is necessary for cell-cycle progression from G0 through G1 to the S phase; while, the binding of cyclin E and the concomitant activation of CDK2 is necessary for entry into S-phase [168].

In NIH 3T3 cells activated Cdc42(G12V ) leads to increased levels of cyclin D. Cyclin D and CDK4 can hyperphosphorylate the retinoblastoma (Rb) tumor suppressor protein that functions to sequester and inactivate E2F transcription factors. The pRB hyperphosphorylation leads to the release of the E2F transcription factors and initiation of S phase [169].

Constitutively active Cdc42(G12V) leads to the expression of elevated levels of cyclin E and the concomitant activation of CDK2[170]. The activated Cdc42(G12V) had no effect on the cyclin dependent kinase inhibitors (CDKI) s $p21^{WAF1}$ or p27KIP. Activated Cdc42 may activate the downstream effector p70 S6 kinase which then drives the transcription of cyclin E and G1 progression [171]. Finally, increased levels of cyclin A are necessary for the S-phase of the cell-cycle. A study using embryonic mouse

fibroblasts that were transfected with activated Cdc42 and grown in soft agar showed increased levels of cyclin A and cell-cycle progression into S-phase[172].

Cdc42 and cellular transformation

Overexpression or activation of Cdc42 can lead to transformation of cells[173, 174]. Transformed cells exhibit some or all of the following characteristics; anchorage independent growth as measured by colony formation in soft agar, loss of contact inhibition judged by foci formation, loss of growth factor dependence with cell growth in serum-free media, and tumor formation *in vivo*. The expression of Cdc42(G12V), a constitutively active mutant, in rat fibroblast cells enabled the cells to grow on soft agar and caused the formation of tumors in nude mice[174]}. Constitutively active Cdc42(G12V) in rat L6 myoblast cells also caused the cells to exhibit a loss of contact inhibition, and loss of dependence on growth factors[173]. Cells from three human rhabdomyosarcoma cells lines from skeletal muscle tumors were found to have increased levels of activated Cdc42 in a pull-down assay in comparison to normal L6 myoblast cells [173].

Cdc42 functions in the Ras oncogene-mediated transformation of mammalian cells. Expression of dominant negative Cdc42(T17N) blocked Ras transformation of NIH 3T3 fibroblasts by inhibiting anchorage independence exhibited by a lack of colony formation in soft agar [174]; while, the expression of constitutively active Cdc42(G12V) in Ras transformed cells resulted in co-operativity that led to the formation of tumorigenic cells in nude mice[143].

Cdc42 is also involved in the mediation of cellular transformation by Dbl oncogene proteins. Dbl proteins are guanine exchange factors, which activate the small GTPase proteins such as Cdc42. A mutant Cdc42(F28L) mimics the effects of a Dbl protein by enhancing rapid GDP/GTP cycling [175]. The expression of the mutant Cdc42(F28L) resulted in the transformation of NIH3T3 fibroblasts. The stable cell lines that expressed Cdc42(F28L) were tumorigenic when they were injected into nude mice [176]. Deletion of amino acids 120-139 which make up the Rho insert region from Cdc42(F28L) did not affect the rapid cycling of GDP/GTP; yet, it prevented the transformation of the NIH3T3 fibroblasts. The Rho insert region may be important in cell growth and cell transformation [177].

Cdc42 activation of specific downstream effector proteins has been implicated in cellular transformation. The serine/threonine kinase PAK4 is an effector molecule for Cdc42. Activated Pak 4 led to the formation of filopodia in fibroblasts. Fibroblasts expressing Pak 4 exhibited a loss of anchorage independence. Cdc42 activation of PAK4 may mediate anchorage-independent growth and transformation of cells [178].

Another downstream effector of Cdc42 is the PAR6 protein. PAR6 is a mammalian homolog of the *C elegans* PAR 6 protein that is involved in establishing cell polarity in the embryo. PAR6 binds to activated Cdc42 and PKCζ. The ternary complex activates PKCζ and results in cell transformation [179].

The γCOP subunit of the seven subunit COP1 coatomer coat complex binds to activated Cdc42. The binding interaction does not occur at the switch 1 domain of Cdc42

but may take place at the dilysine motif present in the Cdc42 carboxyl terminus. Cdc42 is involved in intracellular vesicular trafficking. The expression of the fast GDP/GTP cycling mutant Cdc42(F28L) with a mutation in the carboxyl terminus dilysine motif prevented the transformation of NIH3T3 cells. Activated Cdc42 and vesicular trafficking may be a part of the cellular transformation [180].

Cdc42 is regulated by guanine nucleotide exchange factors, (GEFs), GTPase activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs).Generally, GDIs inhibit of the dissociation of GDP from the Rho GTPase.; however, RhoGDI-1 also facilitates the release of Cdc42 from the membrane and inhibits the release of GTP from the GTP-bound Cdc42. The binding affinities of GDP-bound Cdc42 and GTP-bound Cdc42 are similar for RhoGDI-1[181]. A mutant Cdc42(R66A) with a mutation in arginine 66 in the switch II region abolished the binding of Cdc42 to RhoGDI-1. NIH3T3 fibroblasts expressing the rapid GDP/GTP cycling Cdc42(F28L) mutant were transfected with the Cdc42(R66A) mutant. The cells co-transfected with Cdc42(R66A) and Cdc42(F28L) did not display anchorage independence assayed by colony formation in soft agar. They also did not demonstrate growth factor independence assayed by growth in serum-free media. They were transformation-defective[175]. Rho GDI-1 appears to be both a negative and a positive regulator of Cdc42. As a positive regulator in signaling pathways it maintains Cdc42 in the activated, GTP-bound state and mediates interactions between Cdc42 and cell membranes. The binding of RhoGDI-1 seems to be important for cellular transformation [182].

Growth factors such as hepatocyte growth factor (HGF) and epidermal growth factor (EGF) can activate Cdc42 [183] [184]. Epidermal growth factor also activates Src tyrosine kinase. Src phosphorylates Cdc42 on tyrosine 64 in the switch II domain. This phosphorylation of Cdc42 does not affect GDP or GTP bindng or the binding to downstream effectors; yet, the phosphoylation of Cdc42 on Tyrosine 64 stimulates the binding of Cdc42 to Rho GDI-1. Epidermal growth factor may increase the binding of Cdc42 to Rho GDI-1 which results in cellular transformation [185].

The removal of the Rho-insert region which consists of 13 amino acids (122-134) from the constitutively active Cdc42(Q61L) mutant had no effect on its ability to bind of Cdc42 effector proteins that have a CRIB domain such as Pak [177].

The Rho-insert region of activated Cdc42 was found to bind to an 85 kDa protein, which is a member of the Cool family, p85Cool-1. The Cool proteins contain a SH3 domain, a Dbl homology domain, and a pleckstrin homology domain; though, p85 Cool-1 does not function as a GEF because it contains a unique sequence which lies downstream of the pleckstrin homology domain [186]. Activated Cdc42 bound to p85Cool-1 associates with c-Cbl. Members of the c-Cbl family are the cellular homologs of the retroviral oncogene v-Cbl [187]. The c-Cbl proteins function as E3 ubiquitin ligases which catalyze the ubiquitinization EGF receptors [188]. The association of activated Cdc42 bound to p85 Cool-1 with c-Cbl results in the sequestration of c-Cbl. Sequestration of c-Cbl prevents the destruction of EGF receptors which results in an accumulation of EGF receptors. These receptors are involved in MAP kinase signaling which leads tocellular transformation [189].

Studies involving the examination of human breast cancer cells have found that EGF receptors are present in large numbers in the transformed breast epithelial cells[190] [191] [192]. A Western blot analysis of human breast tumors and corresponding normal mammary gland tissue from the same patients showed an over-expression of activated Cdc42 in the tumor cells [193]. Another study utilized rat mammary adenocarcinoma cells expressing constructs of dominant negative Cdc42. They found that the expression of dominant negative Cdc42 reduced the number of foci and inhibited colony formation in soft agar [194]. At present, very little is known about the Cdc42-mediated transformation of mammary gland epithelial cells.

# Chapter 3: Mouse mammary tumor virus activates Cdc42 leading to filopodia formation, viral egress and transformation

## Introduction

Mouse mammary tumor virus (MMTV) is a beta-retrovirus that infects mouse mammary epithelial cells, inserts into the genome and causes malignant tumors in susceptible mice [195] [196] [197] [18]. Once integrated, MMTV RNA is transcribed in the nucleus and translated by the host as polyproteins. [198] [20] [199] [102]. The newly translated Gag proteins assemble in the cytoplasm to form a capsid shell or an immature "A" particle whereas viral envelope proteins are transported to the plasma membrane via the host secretory pathway [22]. The A-particles then associate with the envelope proteins at the tips of actin-based filamentous projections [23] [147]. It is likely that these projections are similar to the actin rich projections produced by other retroviruses such as the Type C retroviruses p815 and A-2/Cl.2.2 [200] [201].

The association of MMTV with filopodia suggests that actin assembly may be integral to the process of viral exit. This is supported by reports showing that cytochalasin D inhibited late steps in MMTV maturation and production [108]; yet, the mechanism by which these filamentous processes form is not known. One possibility is that the virus might activate the Cdc42/WASP/ARP2/3 pathway which can induce the

polymerization of monomeric actin into filamentous or F-actin bundles known as filopodia [202] [203] [142] [144] [151] [204].

In this study, we show that egress and exit of MMTV replicating in murine mammary epithelial cells depends on the actin cytoskeleton, Cdc42 and WASP. Immobilized PAK binding assays showed that MMTV activates Cdc42, which was retained on the column along with viral Gag protein. Binding between Cdc42 and MMTV Gag was further evidenced by co-localization of Cdc42 with Gag-containing viral particles in the cytoplasm. WASP also co-localized with viral particles and expression of either a dominant negative Cdc42 or WASP prevented filopodia formation, viral egress, and viral release.

Having demonstrated that Cdc42 was an essential component of the viral exit pathway, studies were carried out to compare the effect of viral infection and Cdc42 activity on cell growth. Viral infection conferred on cells the ability to grow in serum free media, to grow on soft agar and to form foci. Identical results were obtained when uninfected cells were induced to express constitutively active Cdc42. On the other hand, a dominant negative Cdc42 blocked growth of virus-infected cells. These results suggest that activation of Cdc42 is both necessary and sufficient to cause transformation of these cells in vitro.

Materials and Methods

**Cells and cell culture**

NMuMG cells (ATCC CRL-1636) an epithelial cell line derived from the normal mammary glands of NAMRU mice that does not express detectable retroviruses[205] and Mm5MT cells (ATCC CRL-1637) an epithelial cell line derived from a mammary gland tumor infected with MMTV [206] were obtained from the American Type Culture Collection (Manassas,VA). NMuMG cells were cultured in Dulbecco's modified Eagle's medium containing high glucose, 10% fetal bovine serum (Atlas Biologicals, Fort Collins, CO), 5μg/ml bovine insulin (Sigma-Aldrich), and 100U/ml penicillin and 100μg/ml streptomycin (Invitrogen). All cell lines were maintained at $37^{o}$ C in a humidified atmosphere with 5% $CO_2$. To induce expression of MMTV, infected cells were treated with 4μM dexamethasone (Sigma-Aldrich) overnight (16 h).

**Creation of a MMTV-infected normal mouse mammary gland cell line**

Uninfected NMuMG cells were freshly-infected with MMTV by co-culturing them with virus expressing Mm5MT cells. To prevent mixing of the two cell lines, Mm5MT cells were grown on 12 mm coverslips which were then transferred to the well of a 0.4-μm pore-size filter insert (Falcon 3090). The insert was then placed in 35 mm culture dishes containing NMuMG cells at a density of $10^3$ cells /35 mm dish in 3 ml of growth media (Figure 9). After 5 days in culture the inserts with the Mm5MT cells were

discarded. The infected NMuMG cells were assayed using immunostaining for MMTV infection with an antibody specific for MMTV p27.

- Plate 10 $^{3}$ un-infected NMuMG cells/35mm well
- Plate 10 $^{2}$ infected cells on 12mm diameter glass coverslips and place in cell culture inserts
- Place inserts in wells
- Add growth media into well with pipette
- Cells co-cultured for 5 days 37$^{o}$ C, 5%C0$_{2}$
- Inserts discarded
- Newly infected cells assayed with anti-MMTV p27
- NMuMG+MMTV cells

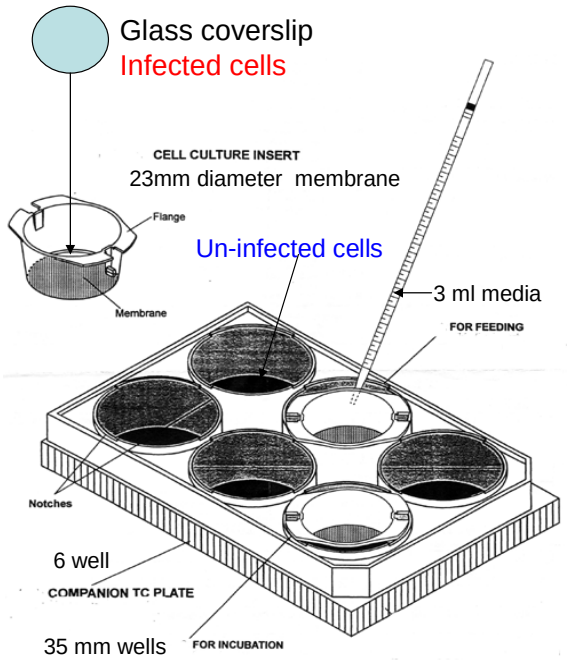


Figure 9. Overview of cell co-culture protocol

## DNA constructs

### *Expression of Cdc42 constructs*

A dominant negative Cdc42 (Cdc42T17N) fused to GFP was placed under the control of a tet- inducible promoter using the Clontech Tet On/Off system vectors (pTet-On, and pTRE2hyg) (Clontech, Palo Alto, CA) (Figures 10 and 11). The GFP-Cdc42T17N was subcloned from pcDNA3GFP-Cdc42T17N (a gift from Dr. Surangani Dharmawardhane, University of Texas). PCR amplification GFP-Cdc42T17N was carried out using 5′-GCGCGGATC CGCCGCCATGA GTAAAGGAGAAGC and 3′-CGCGATCGATTCATAGCAGCA CACACCTGCGGC as primers. The amplified DNA was then placed into pTRE2hyg vector using the BamHI and ClaI sites.

NMuMG or NMuMG+MMTV cells were transfected with the pTet-On plasmid using NovaFECTOR reagent (Venn Nova, Pompano Beach, FL). The cells were selected with 1mg/ml G418 (Invitrogen) to obtain a stable cell line, which was used for subsequent isolation of clones. The cloned cell line was then transfected with pTRE2hyg -GFP-Cdc42T17N or with pTRE2hyg vector only using the NovaFECTOR protocol. NMuMG+MMTV-Tet-On-pTRE2hyg-GFP-Cdc42T17N and NMuMG+MMTV-Tet-On-pTRE2 vector stable cell lines were selected using 1mg/ml G418 and 400μg/ml hygromycin B (Clontech) (Figure 12). The expression of the GFP-Cdc42T17N was confirmed using fluorescence microscopy to compare uninduced cells with those treated with 2μg/ml doxycycline.

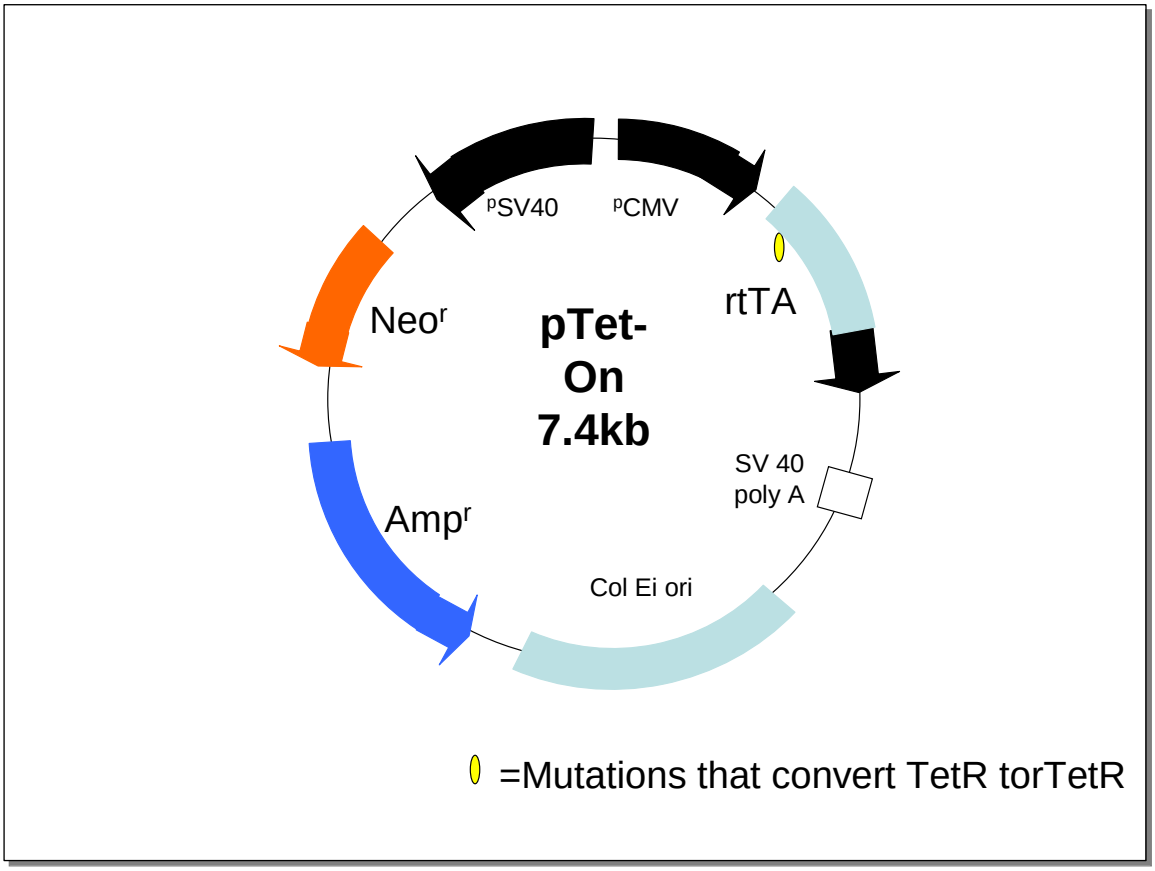


Figure 10. Regulator plasmid for Tet-On gene expression system (Clontech, Palo Alto, CA).

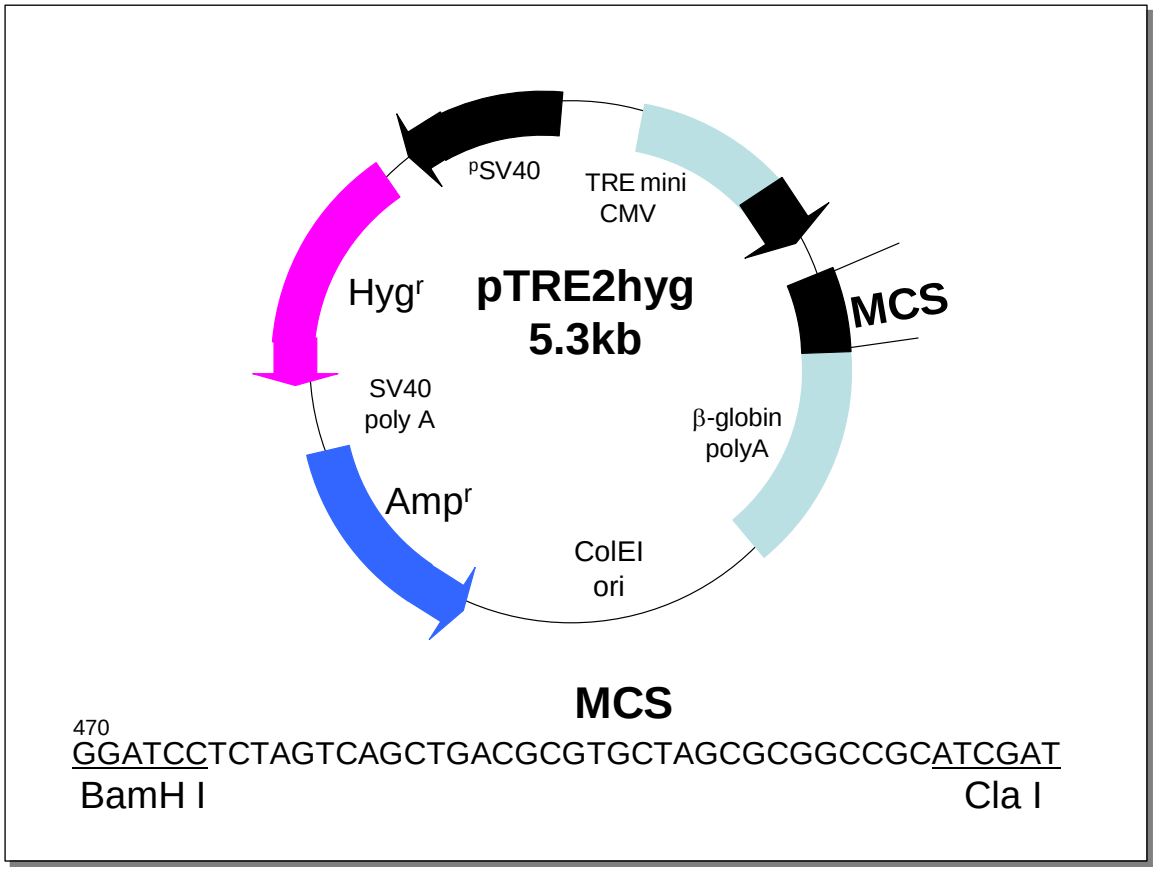


Figure 11. Response plasmid, pTRE2hyg for the Tet-On gene expression system (Clontech, Palo Alto, Ca).

The genes GFP-Cdc42(Q61L) and GFP-Cdc42(T17N) were inserted respectively, into the multiple cloning sites using the *BamHI* and *ClaI* restriction sites.

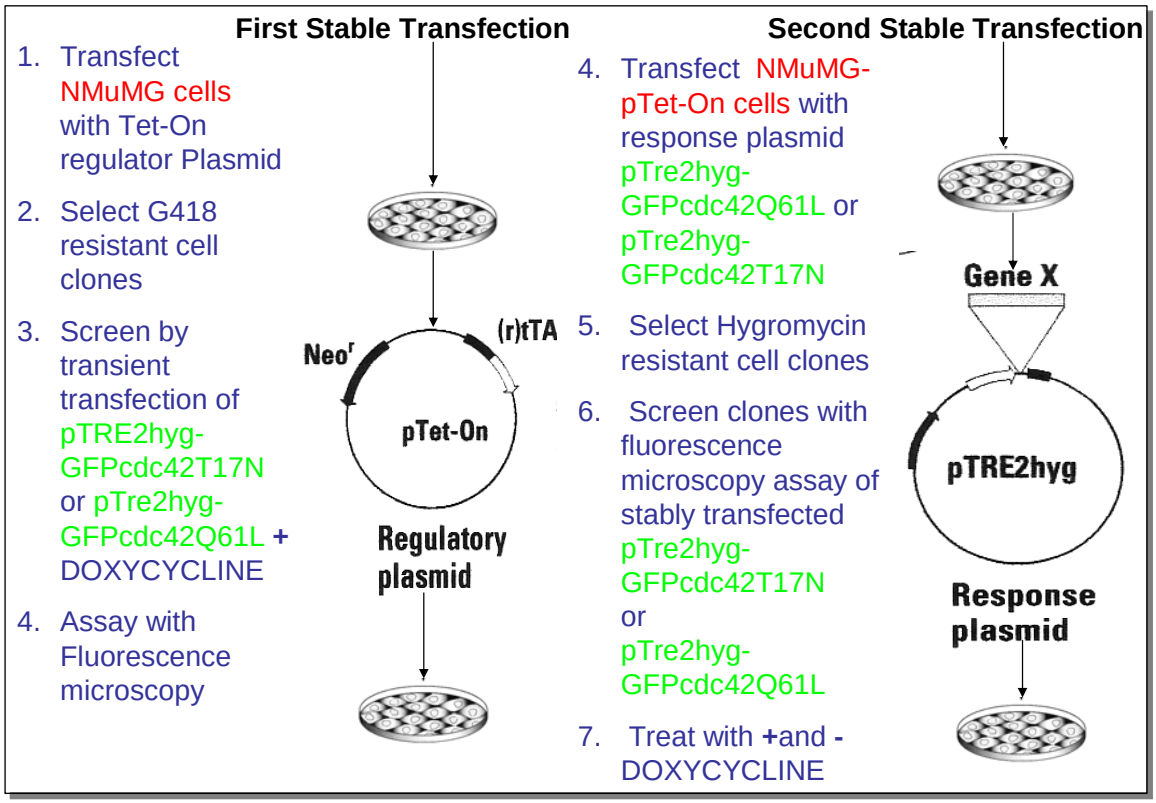


Figure 12. Overview of the development of Tet-On double-stable cell lines

Cells transiently expressing Cdc42WT fused to green fluorescent protein were generated by transfecting NMuMG with pcDNA3GFP-Cdc42WT (a gift from Dr. Surangani Dharmawardhane, University of Texas at Austin) using NovaFECTOR. Cells were transfected at 80% confluence using 1μg plasmid DNA and 5μL Nova FECTOR added to 1 ml of serum and antibiotic free MEM in 35mm plates. After an 8 hour, incubation at 37oC the reaction was quenched by adding 1 ml of DMEM supplemented with 20% fetal bovine serum. After 48 hours, cells were assayed for expression of GFP with fluorescence microscopy.

***Expression of WASP constructs***

WASPWT or a WASP mutant missing the CDC42 binding domain (WASPΔGBD) were subcloned from pQBI25WASPWT and pQBI25WASPΔGBD (gifts from Dr. Don Stewart at the NIH) using PCR with 5′-GTATTCGGTACCA ATGAGTGGGGGCCCAATGGGA and 3′- TCGCTACCGGTGCGTCATCCCATTCATCATCTTC primers containing the KpnI and PinAI restriction sites. Each WASP construct was then spliced into the p-EYFP-1 vector (Clontech) (Figure 13) containing the gene for yellow fluorescent protein. NMuMG and NMuMG+MMTV cell lines were transfected with the WASP constructs by electroporation using 50 μg DNA and an ECM 600 (Harvard Apparatus, Holliston, MA); at 500V, capacitance 2000uF, resistance R4 of 72 ohms, charging voltage of 300V and a pulse duration of 25 milliseconds). After electroporation, cuvettes were incubated for 30 minutes on ice and then transferred to10cm tissue culture dishes. Stable cell lines were obtained by continuous selection with 1mg/ml G418 for 2 weeks. The cells were assayed for expression of EYFP with fluorescence microscopy.

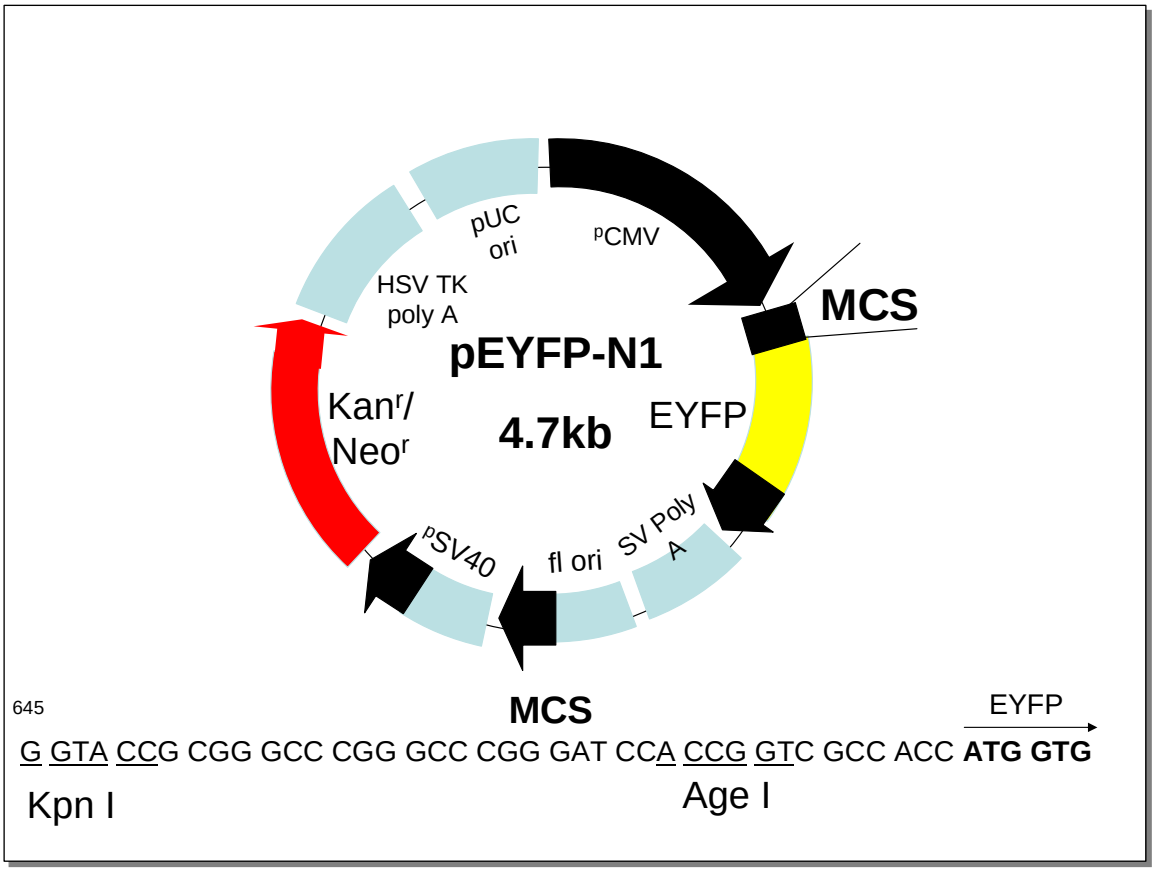


Figure 13. EYFP expression plasmid, pEYFP-N1 (Clontech, Palo Alto, CA).

The plasmid has an enhanced yellow-green variant of the Aequorea victoria green fluorescent protein. The PinAI and KpnI restriction sites in the multiple cloning site were used to incorporate WASP and WASPΔGBD genes into the plasmid.

**Assay for MMTV in supernatants**

Cell cultures were grown to confluence in 35mm culture dishes, rinsed with serum-free DMEM twice and then cultured in 2ml 4μM dexamethasone in serum-free DMEM overnight (16h). Tet-On NMuMG+MMTV cell lines were treated with 2μg/ml doxycycline overnight (16h). Supernatant was removed and put in 20ml glass tubes in a Labconco Lyph-Lock 6 lyophilizer for 3 hours. The dehydrated samples were diluted in 100μl of 1 x Laemmli sample buffer and incubated at 100$^{o}$C for 5 minutes. The samples were analyzed on 12% SDS-polyacrylamide gels and Western blotted using a 1:500 dilution of goat polyclonal anti-MMTV gp52 antibody (a gift from Dr. Jacquelin Dudley, University of Texas)[207]. Proteins were then detected with alkaline phosphatase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA).

**Preparation of cell lysates, electrophoresis and immunoblotting**

The cells were grown to confluence in 100mm plates. The media was removed and the cells were rinsed with ice cold Tris Buffered Saline (TBS) containing, 150mM NaCl and 25mM Tris-HCl adjusted to pH 7.4. The TBS was removed and replaced with 0.5ml of lysis buffer containing 5mM $MgCl_2$, 1% Nonidet-40 (NP-40, Sigma Chemical Co, St. Louis, MO), 1mM dithiothreitol, 1μg/ml aprotinin, 1μg/ml leupeptin and 1mM phenylmethanesulfonyl fluoride in TBS. The cells were scraped off the plate into a 1.5ml

microfuge tube, vortexed and insoluble material pelleted by centrifugation at 16,000 x *g* at 4°C for 20 minutes. A 250µl aliquot of lysate supernatant was removed and diluted in 250µl of 2 x Laemmli sample buffer and heated at 100°C for 5 minutes. The samples were analyzed on 12% SDS-polyacrylamide gels and Western blotted using a 1:500 dilution of goat polyclonal anti-MMTV gp52 antibody. Proteins were then detected with alkaline phosphatase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA).

**Assay for activated Cdc42**

Activation of Cdc42 was assayed using immobililzed GST-CRIB fusion protein (Pierce Biotechnology, Rockford, IL). For the assay, confluent cells plated on 10 cm plates were transferred to serum-free MEM and incubated overnight with or without 4µM dexamethasone.The media was then removed and the cells were rinsed with ice-cold TBS followed by 0.5 ml lysis buffer. The cells were scraped off the plates into a 1.5ml microfuge tubes, vortexed and supernatants isolated by centrifugation at 16,000 x *g* at 4°C for 20 minutes. The protein concentration in each supernatant was quantified using a Bio-Rad Bradford assay (Bio-Rad, Hercules, CA)[208] and the protein concentration was normalized to 1 mg/ml. Some supernatants (0.5 ml) were then incubated with buffer alone or buffer containing either 0.1mM GTPγS or 1mM GDP by rotating at 30°C for 15 minutes. The reaction was terminated with the addition of 1M $MgCl_2$ to a concentration of 60mM $MgCl_2$ and samples were incubated on ice for 5 minutes. Each sample was then incubated with 20µg of the GST-PAK 1 PBD and samples assayed according to the

manufacturer's directions. After washing, spin discs were suspended in 100µl of 1 x Laemmli sample buffer, incubated at 100$^{o}$C for 5 minutes and centrifuged at 7,200 x *g* for 2 minutes. The samples were analyzed on 12% SDS-polyacrylamide gels and Western blotted using a 1:250 dilution of mouse monoclonal $IgG_3$ anti-Cdc42 (Santa Cruz Biotechnology, Santa Cruz, CA) or a 1:50 dilution of mouse monoclonal anti-MMTV Gag (a gift from Dr. Jacquelin Dudley University of Texas)[207]. Proteins were then detected with alkaline phosphatase-conjugated bovine anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA).

**Immunofluorescence microscopy**

Cells were grown on coverslips with or without 4µM dexamethasone treatment overnight (16h) and fixed in 3.75% formaldehyde (Sigma-Aldrich) for 10 minutes. After fixation, cells were permeabilized in 100% MEOH at -20$^{o}$ C for 10 min. For localization of F-actin, coverslips were incubated with 0.5µg/ml rhodamine phalloidin (Sigma-Aldrich) for 1(h) and washed extensively with PBS. Washed coverslips were mounted in Prolong Antifade (Molecular Probes, Eugene, OR). For identification of intracellular MMTV, fixed cells on coverslips were incubated with 1:100 dilution of polyclonal goat anti-MMTV p27 capsid protein ( a gift from Dr. Jacquelin Dudley University of Texas)[207] for 1(h), washed, and subsequently incubated with rhodamine-conjugated bovine anti-goat(Santa Cruz Biotechnology, Santa Cruz, CA) or Alexa Fluor 350-conjugated donkey anti-goat ( Molecular Probes,Eugene,OR). Washed coverslips were mounted in Prolong Antifade.

For localization of endogenous Cdc42, coverslips were incubated with a 1:50 dilution of anti-Cdc42 mAb (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour. After washing, the coverslips were incubated with Alexa Fluor 350-conjugated rabbit anti-mouse IgG (Molecular Probes, Eugene, OR). Washed coverslips were mounted in Prolong Antifade. The prepared slides were examined with a Nikon Eclipse 600 epifluorescence microscope equipped with a Quantrix cooled CCD camera (Photometrics, Tucson, AZ) and MetaMorph digital imaging software (Universal Imaging, West Chester, PA).

**Modulated polarization microscopy**

Cells were prepared for modulated polarization microscopy as described in [109]. Cells $x10^2$ were grown on 25-mm round glass coverslips in growth media. Some cells were stimulated with 4μM dexamethasone overnight (16h). Cells were sandwiched between coverslips in HEPES buffered HBSS and mounted on a stage coverslip holder. Images were obtained using 1.4NA 100x Differential Interference Contrast (DIC) oil immersion condenser and a 1.4 NA 100x (DIC) oil immersion objective lens (Nikon, Inc., Melville, NY) as described previously.

**Soft agar assay**

Cells $x10^3$ cells from the NMuMG, NMuMG-Tet-On-GFP-Cdc42Q61L, NMuMG-Tet-On-GFP-Cdc42T17N, NMuMG+MMTV, and NMuMG+MMTV-Tet-On-GFP-Cdc42T17N cell lines were suspended in 1ml of DMEM plus 10% fetal bovine serum

and 0.33% agarose (Sigma-Aldrich)). Tet-On cell lines were supplemented with 2μg/ml of doxycycline and there was an addition of 5μg/ml bovine insulin to NMuMG cell lines. These cells were plated in a 35mm tissue culture dish on a 2ml layer of solidified 0.5% agarose plus 10% fetal bovine serum. The cells were allowed to settle to the interface between the two concentrations of agarose at 37$^{o}$C for 20 minutes. Then the plates were allowed to harden at room temperature for 30 minutes before they were incubated at 37$^{o}$C in 5% $CO_2$ incubator. They were given 0.5ml of the growth media with the proper supplements for each cell line every 2 days for a period of 14 days. Colonies were counted and photographed under low magnification (X10) with a phase contrast microscope.

**Serum-free growth assay**

Cells x$10^3$ from NMuMG, NMuMG-Tet-On-GFP-Cdc42Q61L, NMuMG-Tet-On-GFP-Cdc42T17N, NMuMG+MMTV, and NMuMG+MMTV-Tet-On-GFP-Cdc42T17N cell lines were grown in serum-free DMEM and plated in 60cm tissue culture plates. Tet-On cell lines were supplemented with 2μg/ml of doxycycline and there was an addition of 5μg/ml bovine insulin to NMuMG cell lines. Fresh media and supplements were exchanged every day for 8 days. Cells were trypsinized and counted with a hemocytometer. Cellular viability was determined by trypan blue exclusion.

**Foci-formation assay**

Cells x$10^3$ from NMuMG, NMuMG-Tet-On-GFP-Cdc42Q61L, NMuMG-Tet-On-GFP-Cdc42T17N, NMuMG+MMTV, and NMuMG+MMTV-Tet-On-GFP-Cdc42T17N cell lines were plated in 35mm tissue culture plates with normal growth media. Tet-On cell lines were supplemented with 2μg/ml of doxycycline and there was an addition of 5μg/ml bovine insulin to NMuMG cell lines. Growth media and supplements were exchanged every two days. After 5 days, the numbers of foci per 35mm plate were counted. Individual foci were photographed under low magnification (X10).

Results

**MMTV-induced changes in cellular morphology in newly infected mouse mammary epithelial cells.**

We previously developed modulated polarization microscopy as a means for imaging the cytoskeleton in living cells and here we sought to apply it as a way to investigate interactions between viruses and the cytoskeleton [109]. For these studies we initially used Mm5MT cells [206] that inducibly express MMTV under control of dexamethasone. The imaging data showed that untreated cells generally had smooth edges but after treatment with dexamethasone, cells contained numerous bright particles. These bright particles were frequently associated with filopodia and were tentatively regarded as viral particles (Figure 14 A and B). Serial images from MPM videos showed that these putative viral particles moved to the cell surface and then would "shoot out" from the tips of filopodia (Figure 14C). Given that filopodia are generated by the polymerization of actin, these data suggested that the virus was able to trigger actin polymerization.

In order to study the interaction of MMTV with the cytoskeleton, strict comparison with uninfected cells required that we generate newly infected cells. For this we used NMuMG cells, a breast epithelial cell line [205]. Since MMTV is poorly infective, we developed a system for co-culturing uninfected NMuMG cells with the C3H strain of MMTV-producing Mm5MT cells using a 0.2μ filter to separate the two cell lines. Using this system, we obtain a large percentage of infected NMuMG cells. To prove that cells were infected after co-culture, we fixed and immunostained the NMuMG

cells with a mAb specific for p27 Gag of MMTV. The results showed that before co-culture, cells had no viral particles present while newly infected NMuMG were loaded with virus (Figure 14 D-F).

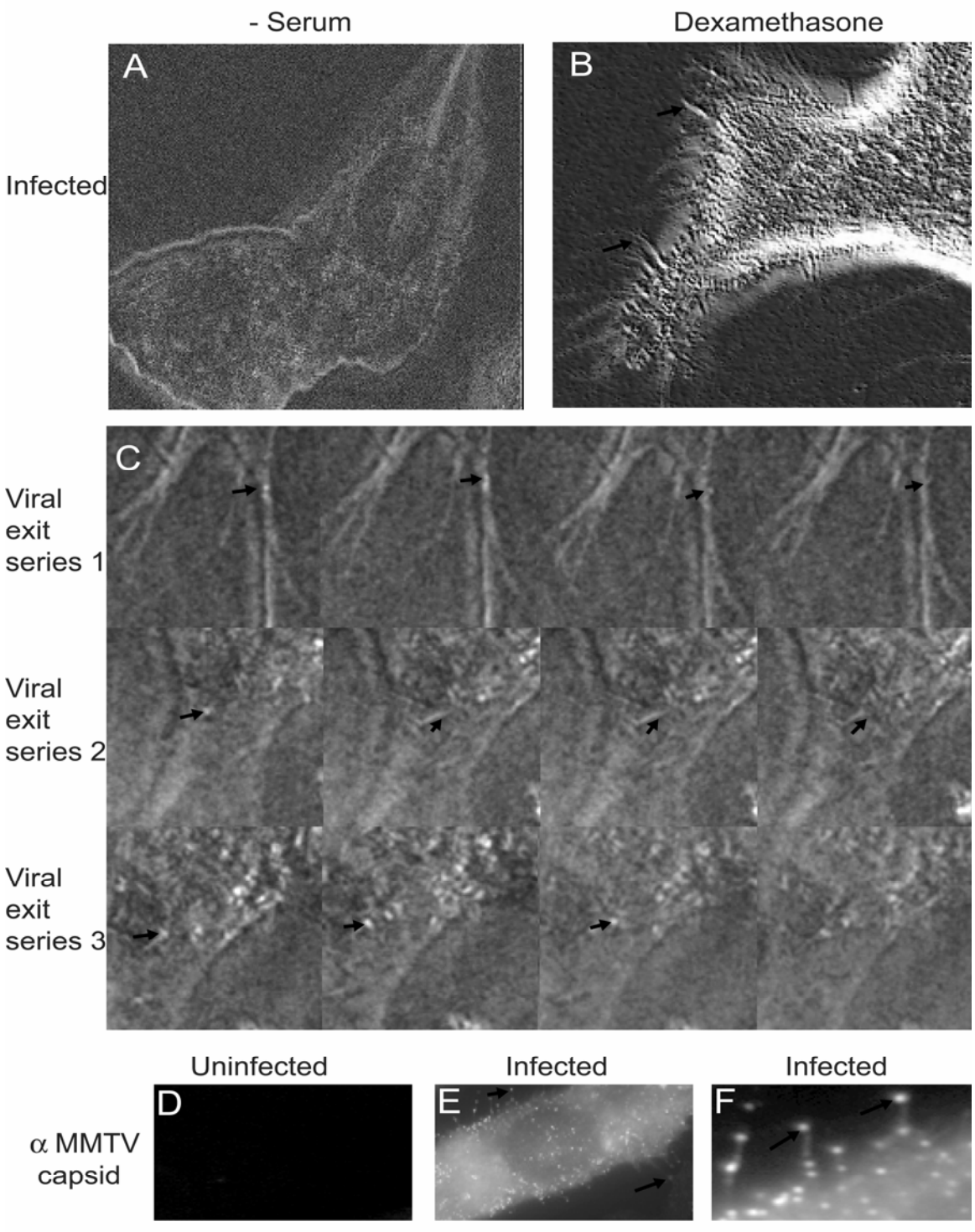


Figure 14. Hormonal induction of MMTV alters the morphology of Mm5MT, mouse mammary tumor cells

Figure 14. Hormonal induction of MMTV alters the morphology of Mm5MT, mouse mammary tumor cells.

Modulated polarization microscopy images of MMTV infected cells.
(A) A serum-starved virally infected Mm5MT cell has a relatively smooth cell membrane.
(B) A virally infected Mm5MT cell induced with 4μM dexamethasone overnight (16h) has many filopodia present on the cell surface. Arrows point to filopodia.
(C) Serial images of MPM videos of infected Mm5MT cells. Putative MMTV particles are ejected from cellular filopodia. Arrows point to putative viral particles.
(D-F) NMuMG cells are newly infected with MMTV. The control uninfected NMuMG cell and the infected NMuMG cell were serum-starved and treated with 4μM dexamethasone overnight (16h), fixed, stained with an antibody specific for MMTV p27 and observed with fluorescence microscopy.
(D) Uninfected NMuMG cell control.
(E and F) Newly infected NMuMG cells have large numbers of MMTV viral particles present in the filopodia. Arrows point to MMTV viral particles.

**MMTV exit from the cell requires an intact actin cytoskeleton**

To determine whether an intact actin cytoskeleton was required for filopodia formation, viral egress and/or exit from the cell, these parameters were monitored in cells treated with cytochalasin B to disrupt actin filaments. For these experiments, MMTV-infected NuMMG were stimulated with dexamethasone for 16hr in the presence or absence and cytochalasin B and then fixed and stained with rhodamine-phalloidin to visualize actin filaments and immunostained with anti-p27 to visualize virus. The results showed that untreated cells contained numerous filopodia that were often associated with viral particles (Figure 15A and C). In the cytochalasin-treated cells, no filopodia were seen and viral particles remained concentrated around the nucleus (Figure 15B and D). These results indicate show that viral egress to the cell surface was dependent on an intact actin cytoskeleton.

Since viral egress was blocked by cytochalasin B, it seemed likely that an intact actin cytoskeleton would also be necessary for viral exit from the cell. To test for the role that actin plays in viral exit, culture supernatants were obtained from dexamethasone-induced infected cells grown in the presence or absence of cytochalasin B. Supernatant from the cultured cells was lyophilized and levels of MMTV virions in each of the supernatants was determined by Western blot analysis using an antibody specific for the MMTV envelope protein gp52. The results showed that cytochalasin B treatment prevented the release of virus from infected cells (Figure 15E).

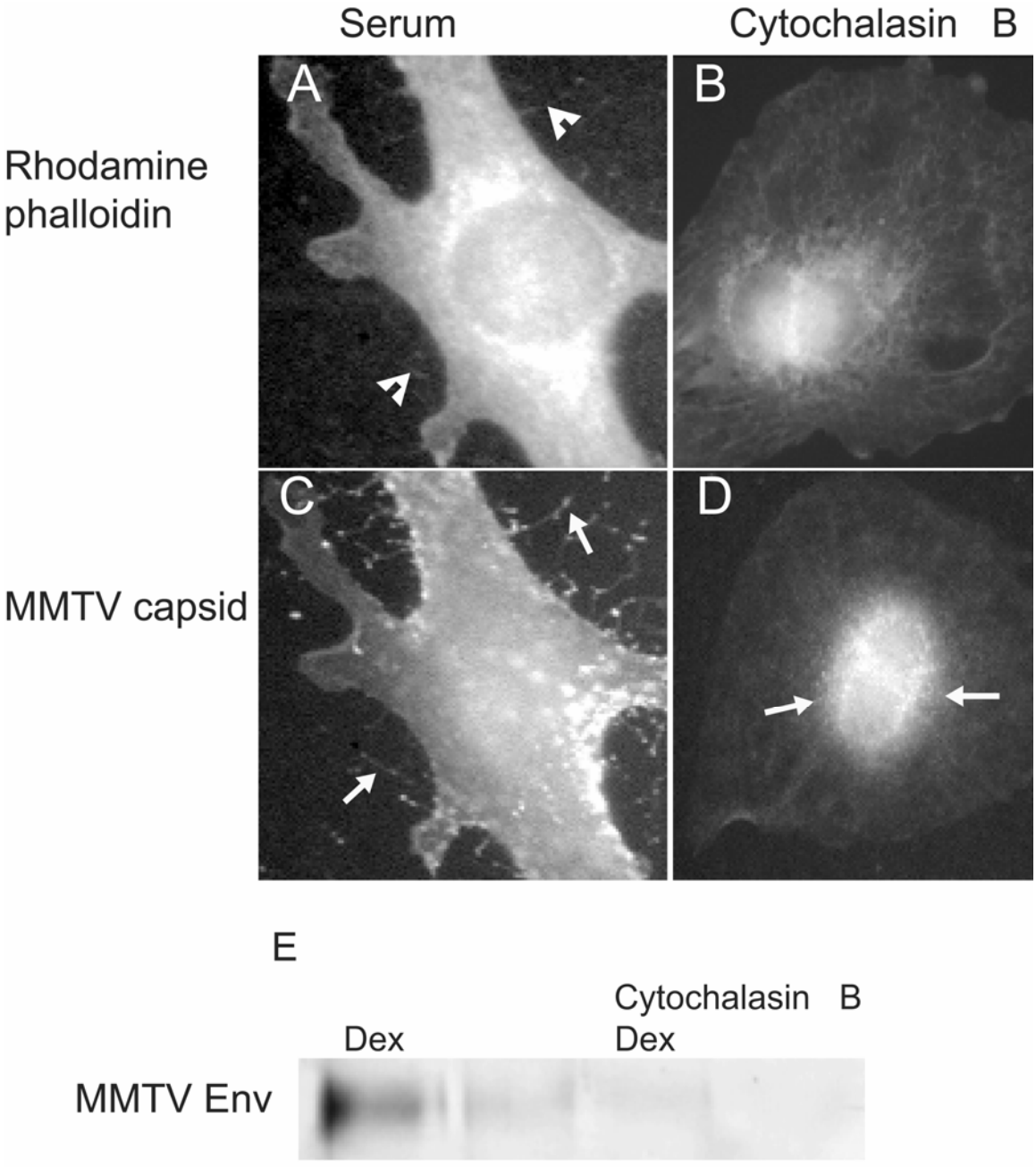


Figure 15. MMTV egress from the cell is an actin-dependent process

Figure 15. MMTV egress from the cell is an actin-dependent process

(A) Infected NMuMG cell serum-starved and treated with 4μM dexamethasone overnight 16(h) and stained with TRITC-conjugated phalloidin. Arrowheads point to filopodia

(B) Infected NMuMG cell serum-starved treated with 4μM dexamethasone and 2.1μM cytochalasin B overnight for (16h) and stained with TRITC-conjugated phalloidin.

(C) Infected NMuMG cell serum-starved, treated with 4μM dexamethasone overnight for 16(h) and stained with an antibody specific for p27 MMTV. Arrows point at viral particles.

(D) Infected NMuMG cell serum-starved treated with 4μM dexamethasone and 2.1μM cytochalasin B overnight 16(h) and stained with an antibody specific for p27 MMTV. Arrows point to viral particles.

(E) Western Blot of supernatants of MMTV infected NMuMG cells grown in the absence or presence of serum, uninduced or induced with 4μM dexamethasone overnight for 16(h),and untreated or treated with 2.1μM cytochalasin B overnight 16(h). Supernatants were probed for mature virions with an antibody specific for MMTVgp52 Env.

**MMTV infection promotes actin polymerization and reorganization of the actin cytoskeleton in mammary cells**

The next question was to determine if expression of MMTV infection induced the formation of filopodia. As controls, uninfected and infected NMuMG cells were grown in serum-free media with or without dexamethasone. Cells were then fixed and stained with rhodamine-phalloidin and analyzed by fluorescence microscopy (Figure 16). The results showed that uninfected NMuMG cells had few filopodia regardless of whether or not dexamethasone was present in the media (Figure 16A, B, and E). Similar results were obtained for MMTV-infected cells in the absence of dexamethasone; however, when infected cells were treated with dexamethasone they formed numerous filopodia (Figure 16F). Similar filopodia were detected when serum-starved uninfected NMuMG cells were treated with bradykinin, an agent known to induce filopodia by activating Cdc42, [142] [209] (Figure 16C).

Since viral infection produced filopodia similar to that seen when cells are treated with bradykinin we were interested to see if expression of a dominant negative Cdc42 would block formation of filopodia. To answer this question, dominant negative Cdc42 was expressed in both uninfected and infected cells NmuMg cells. When these cells were treated with dexamethasone and stained as above, no filopodia were detected in either uninfected or infected cells (Figures 16D and H). These results suggest that MMTV-induced reorganization of the host actin cytoskeleton depends on Cdc42.

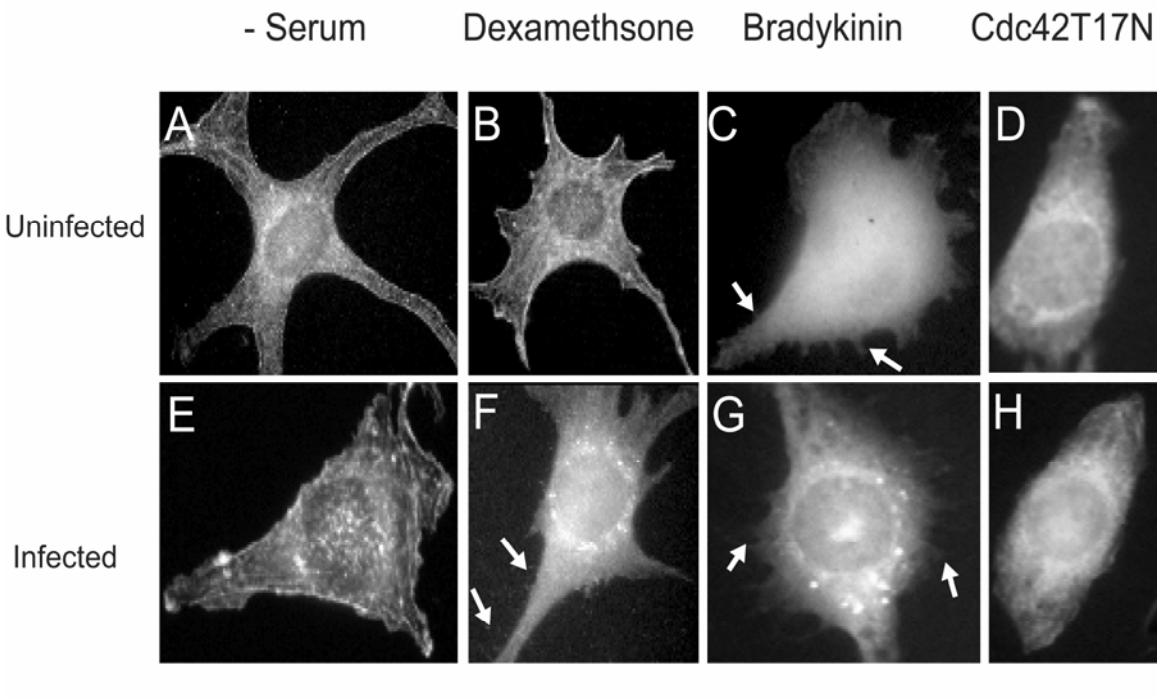


Figure 16: MMTV interaction with NMuMG Cells induces reorganization of the actin cytoskeleton

Serum starved uninfected and infected NMuMG cells were untreated or treated with various reagents at 37$^{o}$C, permeabilized and stained for F-actin with TRITC-conjugated phalloidin.
(A ) Serum starved , uninfected control NMuMG cells.
(B) Uninfected serum-starved NMuMG cells treated with 4μM dexamethasone overnight 16(h).
(C) Uninfected NMuMG cells treated with 100ng/ml bradykinin for 30 min. Arrows point to filopodia
(D) Uninfected NMuMG cells stably transfected with a tetracycline inducible dominant-negative Cdc42 mutant Tet-On-GFP-Cdc42T17N treated with 2μg/ml doxycycline for 24(h) and induced with 4μM dexamethasone overnight 16(h).
(E) Serum-starved infected NMuMG cells.
(F) Infected NMuMG cells, serum-starved and induced with 4μM dexamethasone overnight for 16(h). Arrows point to filpodia.
(G) Infected NMuMG cells treated with 100ng/ml bradykinin for 30 min. Arrows point to filopodia.
(H) Infected NMuMG cells transfected with tetracycline inducible dominant-negative Cdc42 mutant Tet-On-GFP-Cdc42T17N treated with 2μg/ml doxycycline for 24(h) and induced with 4μM dexamethasone overnight 16(h).

**Expression of MMTV activates Cdc42**

Previous data showed that MMTV induced formation filopodia as part of its mechanism for exiting the cell and that Cdc42 was involved. To determine if the induction of MMTV was related to the activation of Cdc42, pull-down experiments with GST-Pak 1 GBD columns were used to monitor the activation state of Cdc42. For the experiments cell lysates were obtained from uninfected or infected NMuMG cells grown in serum-free media in the presence or absence of dexamethasone. The results showed little binding of Cdc42 in the uninfected cells regardless of whether dexamethasone was present or not (Figure 17A). In the MMTV infected cells, some binding of some binding of Cdc42 was seen in either the presence or absence of dexamethasone. More Cdc42 was retained on the column in the dexamethasone- induced cell lysates (Figure 17A).

**MMTV Gag interacts with activated Cdc42**

The relationship between viral expression and Cdc42 activation suggested that one or more of the viral proteins might interact with Cdc42. To investigate this possibility eluates from the GST-Pak1-PBD pull-down experiment column were probed on Western blots with a mouse mAB against MMTV Gag protein. The results showed that a large amount of viral protein was retained on the column along with activated Cdc42 (Figure 17B). Gag protein was not retained on the column when Cdc42 was not activated.

**MMTV co-localizes with Cdc42 in the cell**

The observed (direct or indirect) binding of MMTV Gag to Cdc42 suggested that Cdc42 might co-localize with MMTV viral particles in the cell. This was examined by immunostaining of virus-infected cells with antibodies specific for Cdc42 and p27 Gag. Fluorescence images of these cells showed coincident dot-like staining for both Cdc42 and MMTV throughout the cell (Figure 17C upper panel). Similar data was obtained from cells transfected with GFP-Cdc42WT that were fixed and immunostained for MMTV Gag. Here dot-like staining for MMTV Gag coincided with GFP-fluorescence (Figure 17C lower panel).

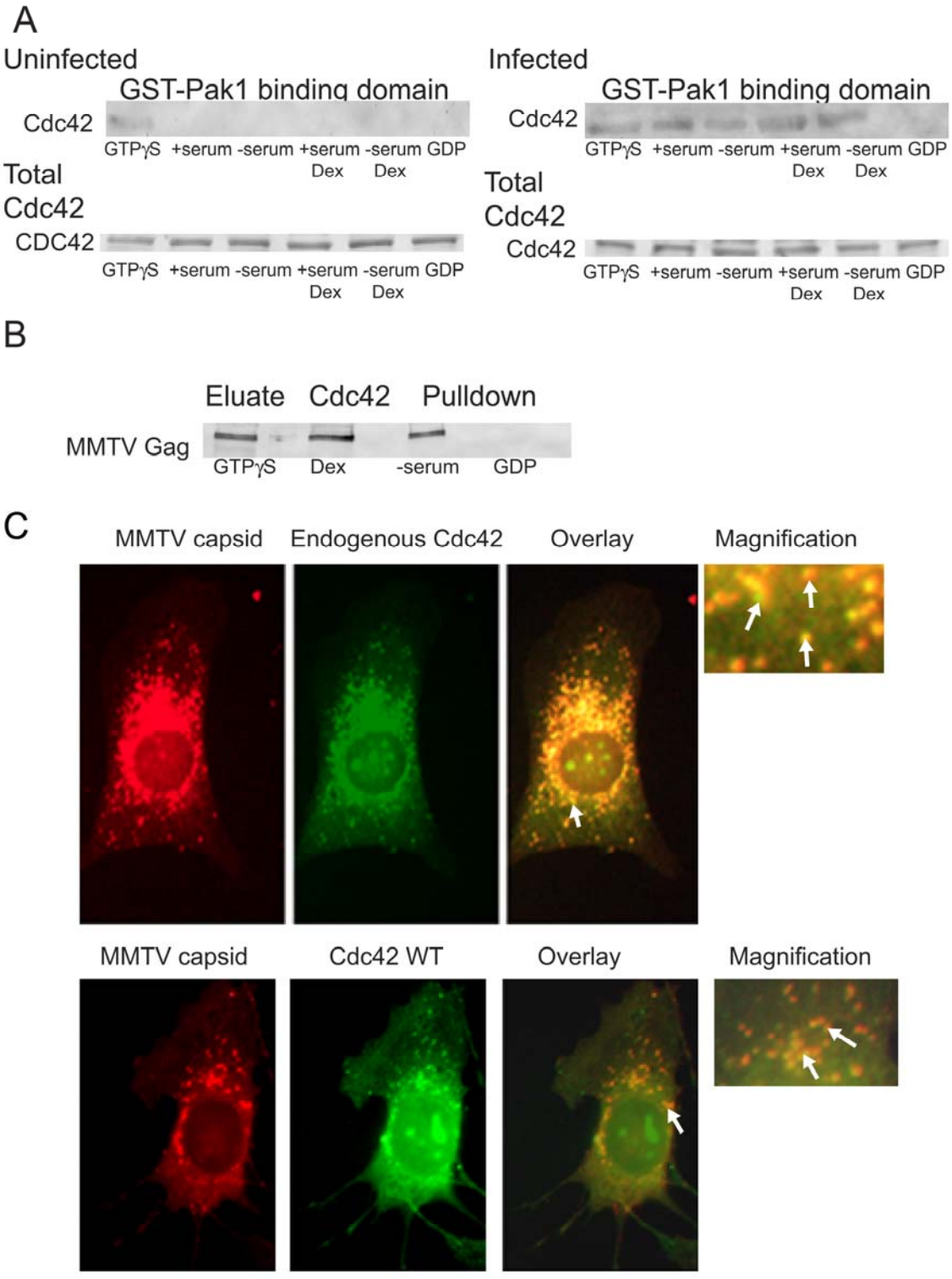


Figure 17. MMTV interacts with activated Cdc42

Figure 17. MMTV interacts with activated Cdc42

(A) MMTV activates Cdc42 in infected NMuMG cells. The amount of activated GTP-bound Cdc42 was determined by GST-Pak 1-PBD pull-down assay which, used 500μg protein extracts and 20μg of the GST-Pak 1-PBD fusion protein.

The loading controls with total Cdc42 content in 500μg protein extracts of uninfected NMuMG cell and infected NMuMG cell lysates were determined by Western blots probed with an antibody specific for Cdc42. GTPγS is the positive control and GDP is the negative control.

(B) MMTV interacts with activated Cdc42 in the eluate from a GST-Pak1-PBD column. Western blot of the Cdc42 pull-down assay eluate probed with MMTV p27 mouse monoclonal IgG antiserum.

(C) MMTV co-localizes with endogenous Cdc42 in the infected NMuMG cell. Fluorescence microscopy of an infected NMuMG cell immunostained with MMTV p27 goat antibody polyclonal antiserum, colored red and with a Cdc42 mouse $IgG_3$ monoclonal antiserum colored green. Arrows point to MMTV co-localized with Cdc42.

(D) MMTV co-localizes with expressed GFP-Cdc42WT in a serum-starved infected NMuMG cell induced with 4μM dexamethasone overnight for 16(h). Fluorescence microscopy detects the fluorescence of transiently transfected GFP-Cdc42WT colored green in the cell. At the same time, the cell is immunostained with MMTV p27 goat polyclonal antiserum colored red. Arrows point to MMTV co-localized with Cdc42.

**MMTV exit from the cell is prevented by dominant negative Cdc42**

Since MMTV appears to activate Cdc42, we next sought to determine if Cdc42 activation was required for viral exit. This was tested by generating an infected NMuMG cell line that expressed a dominant negative GFP-Cdc42 construct (Cdc42T17N) under the control of a tetracycline inducible promoter (Tet-On). Infected cells were grown in the presence of dexamethasone with some cells induced to express Cdc42T17N with doxycycline while parallel samples were not induced. After 16 hours, culture supernatants were harvested, lyophilized and probed on Western blots using an antibody specific for MMTV gp52 Env. The results showed that when dominant negative Cdc42T17N was induced, release of virus from the cell was blocked (Figure 18A). This suggested that activated Cdc42 was needed for viral exit.

**MMTV does not co-localize with dominant negative CDC42 IN the cell**

Previous data showed that MMTV Gag co-localized with Cdc42 in cells and bound directly or indirectly to Cdc42 on PAK 1 PBD columns. In addition, dominant negative Cdc42T17N blocked the release of MMTV from the cell. This raised the question of whether dominant negative Cdc42T17N would co-localize with MMTV in the cell. To answer this question, the NMuMG cells containing Tet-responsive GFP-Cdc42T17N were induced with doxycycline, fixed and stained with an antibody specific

for MMTV p27 Gag. Fluorescence image data showed no co-localization of viral particles and dominant negative Cdc42 (Figure 18B).

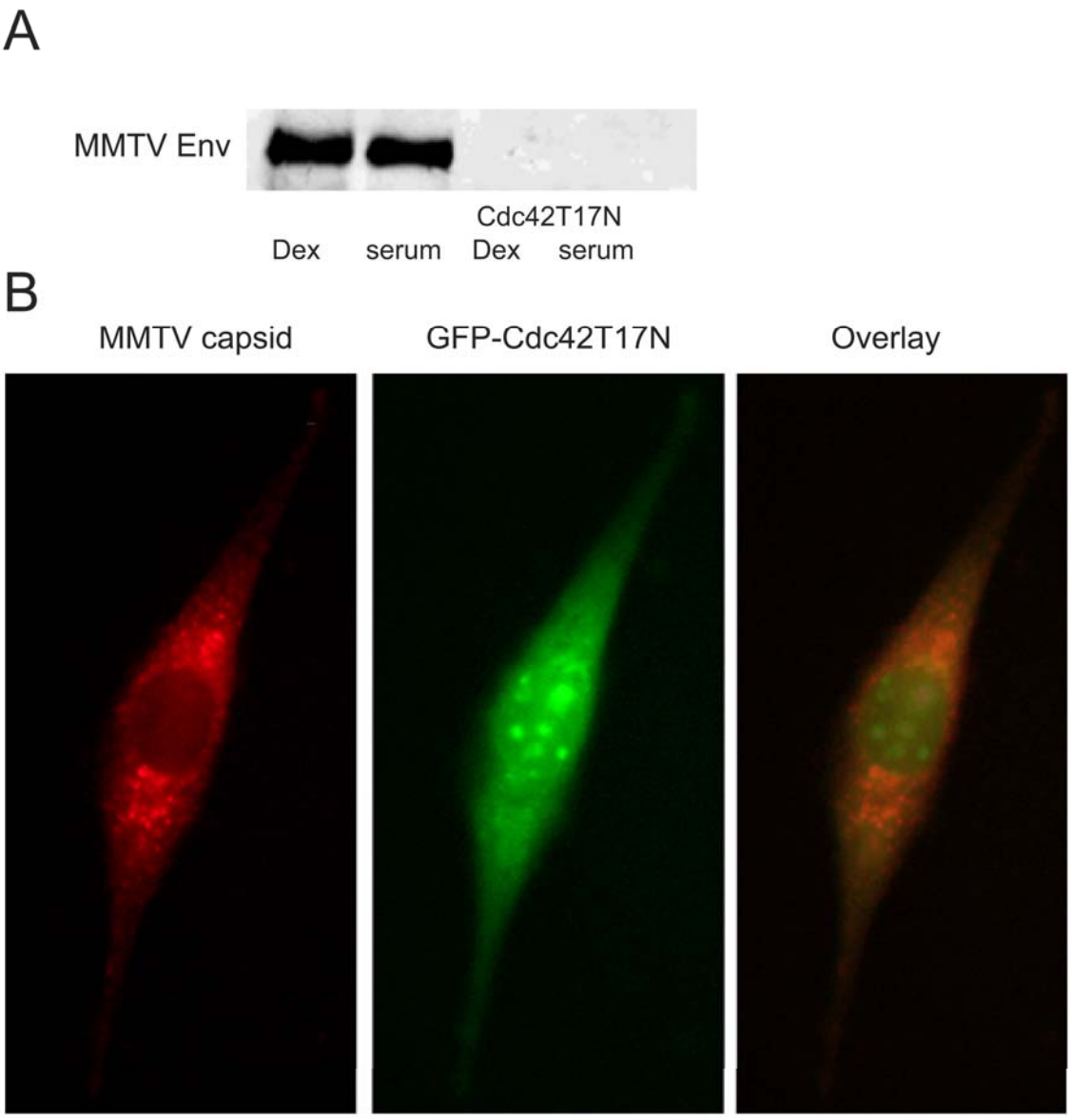


Figure 18. MMTV does not interact with an expressed dominant negative mutant of Cdc42, (Cdc42T17N)

Figure 18. MMTV does not interact with an expressed dominant negative mutant of Cdc42, (Cdc42T17N)

(A) MMTV virions are not released from infected NMuMG cells expressing a dominant negative mutant of Cdc42 (Cdc42T17N). The protein level of MMTV gp52 present in the supernatant of infected NMuMG cells treated with 2μg/ml doxycycline to induce the expression of Tet-On-GFP-Cdc42T17N. The cells were treated with 4μM dexamethasone in the absence of serum to stimulate MMTV production. A Western blot of lyophilized supernatants was probed with anti MMTV gp52 mouse polyclonal antiserum.
(B) MMTV does not co-localize with a dominant negative mutant of Cdc42 (Cdc42T17N). NMuMG+MMTV Tet-On-GFP-Cdc42T17N cells were induced with 2μg/ml doxycycline to express GFP-Cdc42T17N. Fluorescence microscopy detects the flourescence of GFP-Cdc42T17N colored green. The same cell is immunostained with MMTV p27 goat polyclonal antiserum colored red.

**MMTV exit from the cell is dependent on WASP**

Cdc42 controls filopodia formation by binding to its downstream effector WASP that then initiates actin polymerization. This suggested that WASP might also be necessary for viral exit. To test this idea, cell lines were generated that expressed either normal WASPWT or a mutant WASP missing the GTPase binding domain, WASPΔGBD each fused to yellow fluorescent protein (EYFP). The WASPΔGBD construct is known to interfere with actin assembly and filopodia formation [210].

To determine if WASP was necessary for viral exit, cells expressing either EYFP-WASPWT or EYFP-WASPΔGBD were grown in the presence or absence of dexamethasone overnight for 16hr. Culture supernatants were then harvested, lyophilized, and probed on Western blots with an antibody specific for MMTV gp52 Env. The results showed that the expression of EYFP-WASPΔGBD but not normal WASPWT prevented the release of MMTV from the cell (Figure 19A).

**MMTV co-localizes with WASP in filopodia**

Given that MMTV viral particles interact with activated Cdc42, which in turn binds to WASP it seemed possible that WASP might also co-localize with MMTV in the infected cells. To test this idea, infected NMuMG cells, stably transfected with EYFP-WASPWT or EYFP-WASPΔGBD plasmids, were immunostained with an antibody specific for the MMTV p27 Gag. Fluorescence images showed that the fluorescence of EYFP-WASPWT co-localized with MMTV particles in filopodia of cells (Figure 19B).

In cells expressing EYFP-WASPΔGBD no filopodia were present and no colocalization of WASP and virus particles was detected (Figures 19C and D).

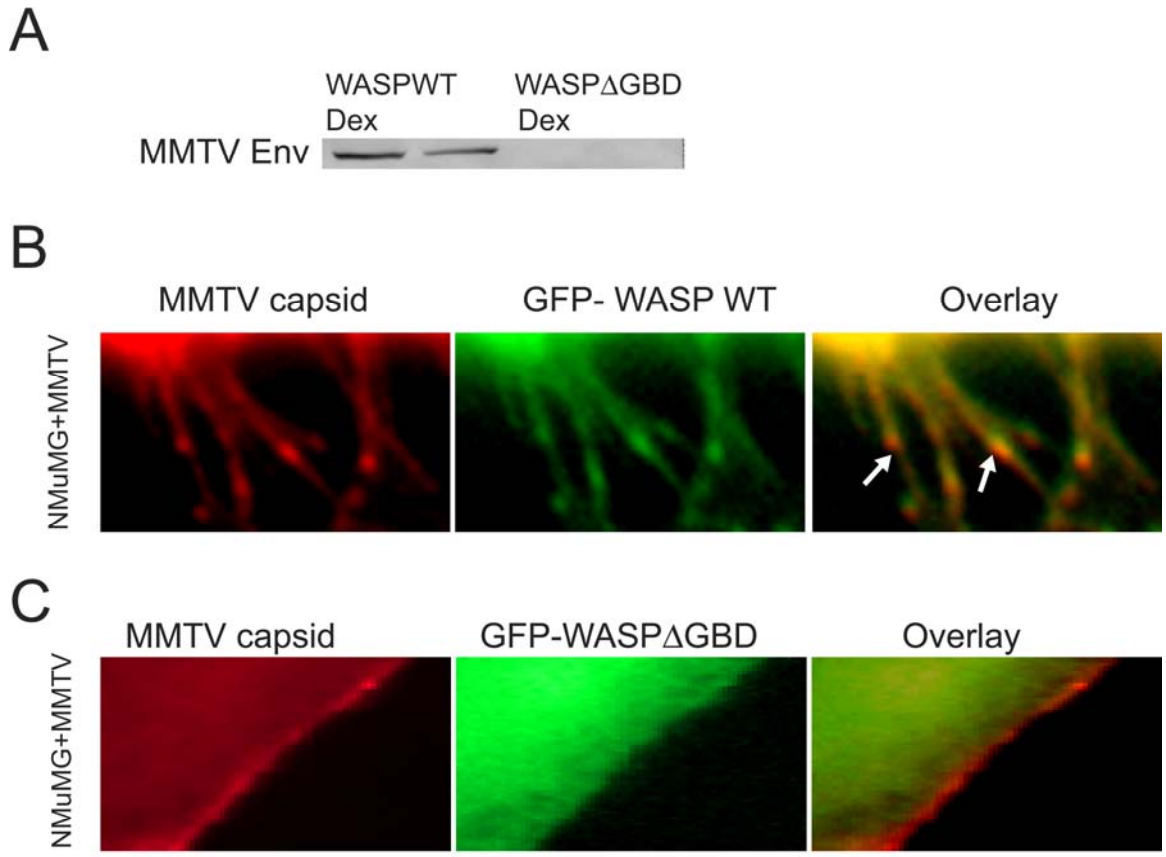


Figure 19. MMTV co-localizes with EYFP-WASPWT for viral egress from the cell.

(A) Lyophilized supernatant of infected NMuMG cells expressing EYFP-WASPWT or EYFP-WASPΔGBD, grown in the presence or absence of 4μM dexamethasone overnight 16(h). Western Blot of lyophilized supernatants probed with MMTVgp52 goat polyclonal antiserum.

(B) Fluorescence microscopy of infected NMuMG cells expressing EYFP-WASPWT colored green and immunostained with MMTV p27 goat polyclonal antiserum colored red.

(C) Fluorescence microscopy of infected NMuMG cells expressing the effector domain mutant EYFP-WASP ΔGBD colored green and immunostained with MMTV p27 goat polyclonal antiserum colored red. Arrows point to virions co-localized with WASP.

**MMTV infection and cell transformation**

Previous results showed that MMTV infection is associated with activation of Cdc42. It has also been found that constitutively active Cdc42 can act as an oncogene [173, 174]. This suggested that MMTV infection might transform cells through the activation of Cdc42. Transformed cells commonly exhibit anchorage independent growth as measured by colony formation in soft agar, loss of contact inhibition judged by foci formation, and loss of growth factor dependence giving cells the ability to grow in serum-free media.

To determine whether MMTV infection could transform cells and if Cdc42 was involved in the transformation process; infected cells were tested for their ability to grow in serum-free medium, to form colonies on soft agar and to form foci. For comparison, identical tests were carried out using uninfected cells expressing constitutively active Cdc42 (Cdc42Q61L) and infected and uninfected cells induced to express dominant negative Cdc42 (Cdc42T17N). The comparisons are shown in Figure 20. The results show that infection with MMTV gives cells the ability to form foci (Figure 20A), to form colonies on soft agar (Figure 20B) and to grow in serum free medium (Figure 20C).

Similar results are seen when uninfected NMuMG cells expressed a constitutively active Cdc42 (Cdc42Q61L) (Figure 20A-C). On the other hand, when infected cells were induced to express a dominant negative Cdc42 (Cdc42T17N) no foci were formed, there were no colonies on soft agar and cells did not grow in serum-free medium much better than uninfected control cells (Figure 20A-C). At a minimum, this suggests that the transforming effects of MMTV viral infection required activation of Cdc42.

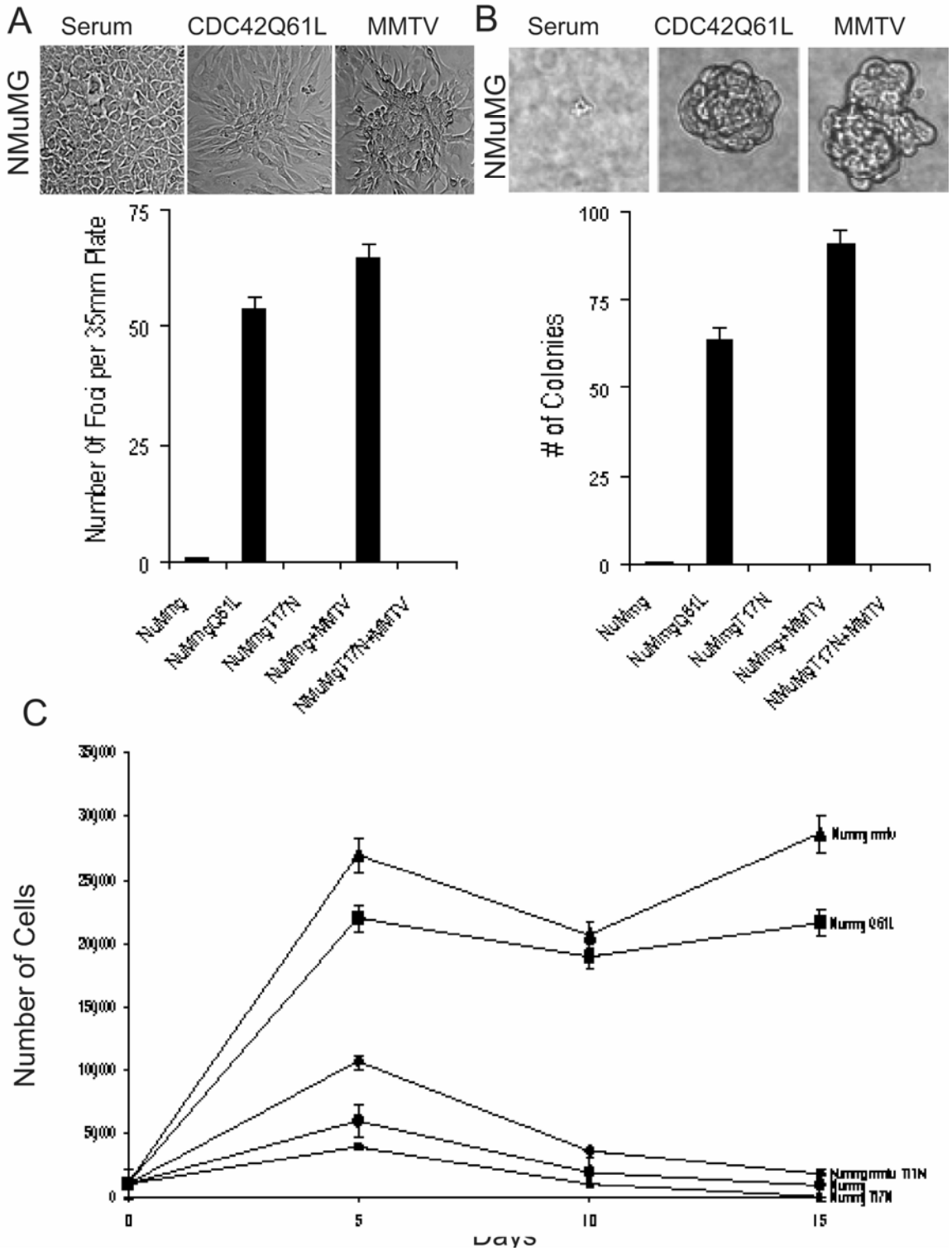


Figure 20. MMTV infection or the expression of a constitutively active mutant of Cdc42, Cdc42(Q61L) transforms NMuMG epithelial cells

Figure 20. MMTV infection or the expression of a constitutively active mutant of Cdc42, Cdc42(Q61L) transforms NMuMG epithelial cells

(A) Uninfected NMuMG cells expressing Tet-On-GFP-Cdc42Q61L and infected NMuMG cells formed foci; however, MMTV infected NMuMG expressing Tet-On-GFP-Cdc42T17N did not form foci. Cells were plated ($10^3$) cells per 35mm culture plate and grown for five days in growth media. Tet-On cells were supplemented with 2μg/ml doxycycline. They were photographed under low magnification (X10). Quantization of foci showed that MMTV infection of NMuMG cells or the expression of Cdc42Q61L in NMuMG cells produced a similar number of foci. Un-infected and infected Tet-On NMuMG cells expressing GFP-Cdc42T17N did not form foci. The experiments were done in triplicate.

(B) MMTV infection or the expression of Tet-On-GFP-Cdc42Q61L in NMuMG cells led to colony formation in soft agar. Uninfected NMuMG cells and MMTV infected NMuMG cells expressing Tet-On-GFP-Cdc42T17N were not able to form colonies on soft agar. Cells ($10^3$) were seeded in 0.33% soft agar media on a layer of 0.50% hard agar media. Tet-On cells were supplemented with 2μg/ml doxycycline. The colonies were photographed under low magnification (X10). The numbers of colonies were recorded 2 weeks after seeding. The experiments were performed in triplicate.

(C) MMTV infection of NMuMG cells or the expression of Tet-On-GFP-Cdc42Q61L in NMuMG cells led to cell growth in serum-free media. Control uninfected NMuMG cells and NMuMG cells expressing Tet-On-GFP-Cdc42T17N exhibited growth factor dependence and were unable to grow in serum-free media. Cells x $10^3$ were grown in serum-free MEM for 15 days. Tet-On cells were supplemented with 2μg/ml doxycycline. The experiments were performed in triplicate.

## Discussion

While some retroviral pathogens exploit the host cellular machinery as part of their life cycle, it is not yet clear how these viruses interact with host signal transduction pathways to promote the reorganization of the actin cytoskeleton [201] [211-213]. Our data indicate that MMTV triggers actin polymerization with subsequent filopodia formation for viral exit in newly infected mouse mammary cells. Previous studies have demonstrated that the organization of the actin cytoskeleton is regulated by cell signaling pathways involving the Rho family small GTPases; Rho, Rac, and Cdc42. Activated Cdc42 induces the formation of actin rich filopodia [209, 214]. To date only two viral pathogens show evidence of Cdc42 activation as a mechanism of triggering actin polymerization [215] [216].

At present, our data indicate that MMTV activates Cdc42, which in turn results in the reorganization of the actin cytoskeleton. Since actin polymerization and filopodia formation are part of the normal MMTV exit pathway, there must be some component of the virus that is directly responsible for activating Cdc42. Thus far, we have found one viral protein, Gag that either directly or indirectly binds to Cdc42. This is seen in the retention of Gag protein along with activated Cdc42 on Pak 1 PBD columns. It is also suggested by the co-localization of Cdc42 and virus seen in the immunofluorescence data. This raises the question of whether Gag might act like a guanine nucleotide exchange factor (GEF) that activates Cdc42.

Gag might act several possible ways to causes nucleotide exchange. One is to mimic the action of the Dbl family of GEFs, which are the largest group of proteins that

activate Cdc42. The Dbl family has a Dbl homology domain (DH) located N-terminal to a Pleckstrin homology domain (PH). The (DH) domain catalyzes the GDP-GTP exchange by binding to the switch 1 and switch 2 region of Cdc42, while the (PH) domain targets Dbl to the cytoskeletal matrix [217]. When Dbl binds to the switch regions, the affinity of Cdc42 for its bound GDP is weakened allowing GDP-GTP exchange.

The fact that Dbl catalyzes nucleotide exchange by binding to the switch regions of Cdc42 makes it an unlikely model for how Gag might activate Cdc42. The reason is that the switch 1 and switch 2 regions that bind to Dbl are the same regions that bind to the Pak 1 PBD column. Yet Gag accumulates on the column, presumably through its binding to Cdc42, even while Cdc42 is bound to the Pak 1 PBD. Thus, Gag cannot bind Cdc42 at the switch 1 and 2 regions. If it were to catalyze nucleotide exchange, it must do so by some other mechanism.

Another possibility is that Gag might activate Cdc42 by mimicking another family of Rho GEFs known as the Dock 180 superfamily [124]. These GEFs lack the Dbl homology domain present in the conventional Dbl GEFs [125]. The protein, Dock 180 has a dock homology region 2 (DHR-2) which has been shown to catalyze the GDP-GTP exchange reaction for proteins in the Rho family [218]; though, Dock-180 only functions as a GEF when it is bound in a ternary complex with Elmo and Crk [127]. Elmo has a (PH) domain, which, targets it to the membrane. The structural data for the Dock 180 complex bound to Cdc42 is not available at this time. The Dock 180 complex may activate Cdc42 through structurally different binding interactions with the switch regions that allow the complex to interact with downstream effectors. One might speculate that Gag could act in a similar fashion.

A final possibility is that MMTV proteins might mimic or trigger the ability of IQGAP to stabilize the activated GTP-bound form of Cdc42[219]. The fact that IQGAP interacts with the actin cytoskeleton[220, 221] and does not interfere with Cdc42 interactions with effector proteins[222] makes this an interesting possibility.

Cdc42 mediates the formation of filopodia by binding and activating WASP. Activated WASP in turn recruits the actin nucleating complex Arp2/3 which induces actin polymerization [202-204, 223] [202]. Studies involving pathogens have shown that the *Shigella* bacterium and the *Vaccinia* virus interact directly or indirectly with a member of the WASP family, N-WASP for the polymerization of actin for the exit of the pathogen from the cell [155, 210].

Our data indicates that activation of WASP is required for MMTV exit and that MMTV co-localizes with endogenous Cdc42 or GFP-Cdc42WT and GFP-WASP in the cell; yet, MMTV does not co-localize with dominant negative GFP-Cdc42T17N or GFP-WASPΔGBD. Since dominant negative Cdc42 and WASP both block viral egress and exit, it is likely that binding to these proteins targets the virus to the cell surface. Furthermore, since actin polymerization is also required for viral egress, it suggests that virus is might be actively driven to the cell surface by actin polymerization. However, we do not find evidence of actin comet tails when infected cells are stained with rhodamine-phalloidin. Thus, the mechanism of viral egress is not clear.

The fact that MMTV activates Cdc42 suggests a new mechanism involved in the transformation of normal mammary epithelial cells. Traditionally, MMTV has been considered an insertional mutagen that sometimes activates the expression of the cellular proto-oncogenes of the *Wnt* family, *Wnt-1* [35], *Wnt-3* [36] *Wnt-10b* [37]; the fibroblast

growth factor family *Fgf-3/int-2* [38], *Fgf-4/hst* [39], *Fgf-8/AIGF* [40], *Fgf10* [41]; the *Notch* family *Notch-4/int-3* [42]; or the *int-6* the gene encoding the p48 subunit of the eukaryotic translation initiation factor eIF3 [43]. It should be noted that insertion of MMTV proviral DNA into the chromosomes of mouse mammary cells is still a random process[224] [19]. In addition, a large percentage of mammary tumors occur without detectable MMTV proviral integrations near the *Wnt, Fgf, or Notch* family loci [69] [67, 68, 225]. This and data from mammary epithelial cell lines transfected with expression vectors encoding the *Wnt-1* oncogene showing that the *Wnt-1* insertion is not directly tumorigenic suggests that other factors might be involved in MMTV tumorigenesis [226]. We were in particular interested in the possibility that viral activation of Cdc42 might transform cells and perhaps serve as an early component of a multistep pathway to tumor formation [227]. Given that, Cdc42 is oncogenic in some cell types [174] [173, 193, 228] it might also cause cellular transformation of normal mouse mammary cells.

To test for a role of Cdc42 in MMTV induced transformation we freshly infected NMuMG cells. Since these cells were derived from normal mammary tissue of the NAMRU strain of Swiss Webster outbred mice it would avoid the biased insertion frequencies seen in highly inbred mouse strains [205]. Furthermore, freshly infected cells avoid the long-term selection and possible accumulation of genetic defects in long-term cell lines harboring MMTV. Newly infected cells from the outbred NAMRU mouse strain would have many, varied, random insertions of the MMTV provirus into the host genome.

We found that newly MMTV-infected NMuMG cells plated at a density of $10^3$ cells per plate exhibited a transformed phenotype that included growth in serum-free

media, colony formation on soft agar, and the formation of foci. None of these features was detected in uninfected cells. In contrast to the massive die off seen in uninfected control cell lines, we observed continuous growth of infected cells in serum-free media. This makes it unlikely that cell growth depended on a particular viral insertion. In addition, many colonies were observed when freshly infected cells were plated on soft agar at a density of $10^3$ cells per 60mm plate. These results suggest that transformation depended on the activation of varied cellular oncogenes. Notably, we also found that that expression of constitutively active Cdc42 (Cdc42Q61L) in uninfected NMuMG cells gave results that were nearly identical to that seen in the MMTV-infected cells. Furthermore, expression of dominant negative Cdc42 (Cdc42T17N) in infected NMuMG cells blocked the ability of MMTV to transform cells. Thus, it appears that activated Cdc42 is sufficient to transform mammary cells.

The data presented here provide a novel mechanism for viral egress and exit as well as a novel mechanism for viral transformation yet much remains to be clarified. If the viral Gag protein functions as a GEF, it may be by a new mechanism that differs from traditional GEFs. Although activation of Cdc42 triggers actin polymerization and the actin cytoskeleton is needed for both viral egress and exit, we have found no evidence of cytoplasmic actin tail formation as seen with *Listeria, Shigella*, and *Vaccinia* infected cells [229] [230] [231]. Thus it is not clear how actin is being employed to move the immature viral "A" particles out to the cell periphery.

Viral activation of Cdc42 may also be relevant to human breast cancer. Several studies report the presence of MMTV DNA sequences of endogenous or exogenous origin in human breast tumor tissue but not in surrounding normal cells[72] [232].

Furthermore, Cdc42 is commonly activated or overexpressed in human breast tumors [193, 228]. Thus, the elucidation of interactions between the host Cdc42 signal transduction pathway and MMTV viral genes could be important some forms of human breast cancer.

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# Vita

Gail Perry Smith was born in San Antonio, Texas the daughter of Marion Elizabeth Perry and Dr. Richard Lacey Perry. She received a degree of Bachelor of Science from Skidmore College. In 1979 she received a degree of Master of Science from New York University Graduate School of Arts and Science. She taught biology for high school students in Huntington, New York and Austin, Texas. In August 1998, she entered the Graduate School College of Natural Sciences of the University of Texas at Austin. She joined the laboratory of Dr. Martin Poenie in 1998. She has been a teaching assistant for the classes Introduction to Cell Biology, Introduction to Genetics, Cell Biology, Graduate Cell Biology, Human Embryology, Introduction to Cell and Molecular Biology Laboratory, Molecular Biology Laboratory, Histology Laboratory, and Anatomy and Physiology Laboratory.

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