

Chemical genetic analyses of quantitative changes in Cdk1 activity during the human cell cycle

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7 **Chemical genetic analyses of quantitative changes in Cdk1 activity during the**
8 **human cell cycle.**
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Abstract

Cyclin dependent kinase 1 (Cdk1) controls cell proliferation and is inhibited by promising anticancer agents, but its mode of action and the consequences of its inhibition are incompletely understood. Cdk1 promotes S and M-phases during the cell cycle but also suppresses endoreduplication, which is associated with polyploidy and genome instability. The complexity of Cdk1 regulation has made it difficult to determine whether these different roles require different thresholds of kinase activity and whether the surge of activity as inhibitory phosphates are removed at mitotic onset is essential for cell proliferation. Here we have used chemical genetics in a human cell line to address these issues. We rescued cells lethally depleted of endogenous Cdk1 with an exogenous Cdk1 conferring sensitivity to one ATP analogue inhibitor (1NMPP1) and resistance to another (RO3306). At no 1NMPP1 concentration was mitosis in rescued clones prevented without also inducing endoreduplication, suggesting that these two key roles for Cdk1 are not simply controlled by different Cdk1 activity thresholds. We also rescued RO3306-resistant clones using exogenous Cdk1 without inhibitory phosphorylation sites, indicating that the mitotic surge of Cdk1 activity is dispensable for cell proliferation. These results suggest that the basic mammalian cycle requires at least some qualitative changes in Cdk1 activity and that quantitative increases in activity need not be rapid. Furthermore, the viability of cells that are unable to undergo rapid Cdk1 activation, and the strong association between endoreduplication and impaired proliferation, may place restrictions on the therapeutic use of Cdk1 inhibitor

Introduction

Cyclin dependent kinase 1 (Cdk1, Cdc2, Cdc28) is a key cell cycle regulator that is required for cell proliferation. Small molecules that inhibit Cdk1 are potential anticancer agents (1-5) but their modes of action are unclear, partly because they lack complete specificity but, more importantly, because of our incomplete understanding of the way in which Cdk1 activity controls cell cycle progression.

Cdk1 has three key roles in the cell cycle: promotion of S and M-phases, and suppression of replication origin licensing during G2. Failure of the latter role leads to endoreduplication (consecutive S-phases without an intervening M-phase). To achieve such different roles Cdk1 must phosphorylate different sets of substrates at different phases of the cell cycle (6,7). In principle, these different sets may respond either to different Cdk1 specificities, achieved by association of Cdk1 with different cyclins, or to different threshold levels of a single Cdk1 specificity as it rises and falls with each cycle (8). Support for the latter (quantitative) model was recently provided by work in fission yeast showing that a single Cdk1-cyclin combination can fully support cell proliferation (9). Furthermore, using chemical genetics (10), Cdk1 was rendered analogue-sensitive (AS; specifically sensitive to the ATP analogue 1NMPP1) by introducing an F80G mutation into its ATP binding site (9). In this way, varying the concentration of inhibitor experimentally separated the different roles for Cdk1. The extent to which threshold values of Cdk1 activity similarly control the cell cycle in higher eukaryotes remains unclear.

In higher eukaryotes there are multiple Cdk paralogues but, apart from Cdk7, which activates Cdk1 (11,12), Cdk1 is the only Cdk required for viability of cells, or indeed animals (13-15). These results reflect essential roles for Cdk1 in mitosis and suppressing endoreduplication that, in the absence of Cdk1, are not adopted by Cdk2

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3 or any other Cdk paralogue. Depletion or inactivation of Cdk1 therefore prevents
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5 mitosis and, in many though not all cell types, promotes endoreduplication (16-18),
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7 leading to polyploidy and genome instability (19,20). The role for Cdk1 in promoting
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9 S-phase has also been conserved, but is shared with Cdk2 and only becomes essential
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11 when Cdk2 is absent. Thus DT40 chicken B cells lacking functional *CDK1* and *CDK2*
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13 alleles were shown to require exogenous Cdk1 for both S-phase and M-phase (21).
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15 Furthermore, by use of an exogenous Cdk1 carrying an F80G mutation (Cdk1-AS),
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17 the latter study found that low and high concentrations of 1NMPP1, respectively,
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19 caused cell cycle arrest in G2 and G1, consistent with the quantitative model of Cdk1.
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21 Cdk1 inactivation in DT40 cells does not cause endoreduplication, however, making
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23 DT40 cells unsuitable for assessing how endoreduplication responds to quantitative
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25 changes in Cdk1 activity. Inhibition of Cdk1 in other cell types can be achieved by
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27 use of ATP analogues such as RO3306 that preferentially inhibit Cdk1 (22), but their
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29 ability to inhibit other kinases is a concern. There is therefore a need to extend
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31 chemical genetic analyses of Cdk1 to other cell types.
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36 Natural quantitative variations in Cdk1 activity are determined not only by the
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38 abundance of its cyclin partners but also by its phosphorylation status (23-26). Thus
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40 Cdk1 activity at the G2/M transition undergoes a sharp increase reflecting feedback
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42 mechanisms for removing its own inhibitory phosphates at residues T14 and Y15.
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44 This highly conserved biphasic switch mechanism (27,28) is widely believed to be
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46 essential for cell proliferation because interfering with it has been found to cause
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48 aberrant mitosis or cell cycle oscillations (29-37). Exogenous Cdk1 carrying T14A
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50 and Y15F mutations (Cdk1-AF) can, however, rescue a human cell line lethally
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52 depleted of endogenous Cdk1 (38,39) and Cdk1-AF was recently shown to support
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54 cell proliferation in fission yeast (9). Further investigation is therefore needed to test
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3 the possibility that inhibitory phosphorylation of Cdk1, and therefore the G2/M surge
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5 in Cdk1 activity, is not required for the basic cell cycle in higher eukaryotes.
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7 Here we have established a chemical genetic system for analysing Cdk1
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9 activity in a human fibrosarcoma cell line (HT2-19) known to endoreduplicate when
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11 Cdk1 is depleted. We have used this system to investigate how quantitative variations
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13 in Cdk1 activity, including the G2/M surge, affect the cell cycle.
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Results

Xenopus Cdk1-as rescues HT2-19 cells and confers 1NMPP1 sensitivity.

In the parental HT2-19 cells used for these studies, one *CDK1* allele is disrupted and the other is repressed by the *lac* repressor (40). HT2-19 cells therefore depend on the inducer IPTG for viability and undergo endoreduplication and apoptosis in its absence (40). To generate 1NMPP1-sensitive cell lines, HT2-19 cells were transfected with plasmids encoding PAC (puromycin N-acetyl-transferase) and either wild-type or F80G-mutated *Xenopus laevis* Cdk1 (xCdk1-WT or xCdk1-AS), as previously used in chicken B cells (21). xCdk1-AS, but not xCdk1-WT, rescued HT2-19 cells from IPTG-dependence and puromycin-sensitivity in a manner that was completely sensitive to 1NMPP1 (Table 1). Several puromycin-resistant, IPTG-independent clones were isolated and screened for sensitivity to 10 μ M 1NMPP1 (Table 2). Because the xCdk1 expression plasmids linked xCdk1-coding DNA to a GFP reporter gene, clones were also screened for GFP as an initial measure of xCdk1 expression. None of the xCdk1-WT-rescued clones (WT1 to WT7) showed sensitivity to 1NMPP1, regardless of GFP expression levels, whereas all xCdk1-AS-rescued clones (AS1 to AS13) were analogue-sensitive in the absence of IPTG. Interestingly, all clones except AS5 expressed detectable levels of GFP and were sensitive to 1NMPP1 even in the presence of IPTG, suggesting that robust expression of xCdk1-AS confers dominant 1NMPP1-sensitivity.

More detailed analyses of clones AS1, AS4 and AS5 confirmed that all ceased to proliferate and died in response to 1NMPP1 (Figure 1A), undergoing endoreduplication (Figure 1B), so phenocopying parental HT2-19 cells deprived of

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3 IPTG (40). As expected, an xCdk1-WT-rescued clone (WT1) showed no such
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5 response. The dominant effect of xCdk1-AS in clones AS1 and AS4 was also
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7 confirmed, IPTG failing to rescue them from 1NMPP1-sensitivity as it did in AS5.
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9 Immunoblots with an antibody recognising both hCdk1 and xCdk1, or an antibody to
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11 the myc-tag on xCdk1, confirmed that xCdk1 was less abundant in AS5 than in AS1,
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13 AS4 and WT1, but still more abundant than endogenous IPTG-induced hCdk1
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15 (Figure 2 A, B). Flow cytometry and immunoblots with anti-GFP antibody confirmed
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17 that relative levels of GFP expression approximated those for xCdk1 expression,
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19 although xCdk1 was clearly detectable in AS5 while GFP was not (Figure 2 B, C).
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21 Together, these results show that xCdk1-AS can substitute for hCdk1 and confer
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23 1NMPP1-sensitivity, and that such sensitivity is dominant over 1NMPP1-insensitive
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25 hCdk1 unless xCdk1-AS is expressed at physiological levels.
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30 Experiments similar to those described in Table 1 and Figure 1 were also
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32 carried out using expression vectors for human Cdk1 carrying mutations F80G or
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34 F80A (Supplementary Tables S1 and S2), but these yielded clones with only modest
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36 sensitivities to ATP analogues (e.g. Supplementary Figure S1) that were not analysed
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38 further.
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43 **Cells expressing xCdk1 are resistant to the Cdk1 inhibitor RO3306**

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45 As expected from studies in other cell types (17,22), the Cdk1 inhibitor RO3306
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47 induced endoreduplication in parental HT2-19 cells (Figure 3A). Unexpectedly,
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49 however, we found that RO3306 did not induce endoreduplication in AS5 cells
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51 (Figure 3A). Similarly, growth curves showed that AS5 cells, though fully sensitive to
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53 1NMPP1, were resistant to RO3306 (Figure 3B). Furthermore, WT1 cells were also
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55 resistant to RO3306 (Figure 3B). These results suggest that cellular xCdk1, unlike
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3 hCdk1, is resistant to RO3306, at least under the conditions used here. Importantly,
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5 they also show that residual hCdk1 in IPTG-independent HT2-19 derivatives, such as
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7 AS5, is unnecessary for the rescue of Cdk1-depleted cells by xCdk1.
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10 11 **Titration 1NMPP1 does not induce stable G2 arrest in AS clones**

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14 If the amount of Cdk1 activity required to promote mitosis is greater than the amount
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16 required to prevent endoreduplication, it should be possible to find an intermediate
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18 concentration of 1NMPP1 that prevents Cdk1 from promoting mitosis while still
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20 allowing it to suppress endoreduplication. Under such conditions cells are expected to
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22 accumulate in G2 with a 4N DNA content and with no accompanying accumulation
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24 of higher ploidies. To test this we treated xCdk1-AS-rescued clones with a range of
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26 1NMPP1 concentrations and measured the effect on DNA content. Arrest in G2
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28 without endoreduplication will increase the 4N/2N ratio without increasing the 8N/2N
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30 ratio. Primary data (Supplementary Figure S2) were therefore analysed to determine
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32 the 4N/2N and 8N/2N DNA content ratios (Figure 4A). In all clones, except the WT1
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34 control, the 4N/2N ratio was increased by treatment with the higher concentrations of
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36 1NMPP1 but this was always accompanied by parallel increases in the 8N/2N ratio,
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38 indicating that no stable G2 arrest occurred. All three AS clones, despite their
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40 different doubling times and kinetics of endoreduplication, appeared to have a
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42 threshold of ~100 nM 1NMPP1, above which endoreduplication was induced. This
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44 threshold was not sharp: above it the extent of endoreduplication increased gradually
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46 with increasing 1NMPP1 concentrations, while below it low levels of
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48 endoreduplication could still be detected at the latest time points. Similarly, increasing
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50 1NMPP1 concentrations between 0 and 310 nM resulted in increasingly delayed
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52 impairments to cell proliferation (Figure 4B). Thus, although a main threshold at
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3 around 100 nM 1NMPP1 is detectable, reducing the amount of Cdk1 activity appears
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5 to reduce the probability of entering mitosis while increasing the probability of
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7 undergoing endoreduplication over a wide range of Cdk1 activities.
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10 11 **Rescue of HT2-19 by xCdk1-AS with T14A/Y15F mutations**

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14 To test whether inhibitory phosphorylation sites are essential for complementation by
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16 xCdk1-AS, T14A and Y15F mutations (AF) were introduced into the xCdk1-AS
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18 expression plasmid. When transfected into HT2-19, this construct did not generate
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20 IPTG-independent HT2-19 clones (Supplementary Table S2). We reasoned that this
21
22 might reflect Cdk1AF expression that was too robust, as this is known to promote
23
24 premature and lethal entry into mitosis (30,33,35). We therefore replaced the
25
26 powerful CMV promoter driving xCdk1 with the human CDK1 promoter region.
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30 When HT2-19 cells were transfected with this modified construct, IPTG-independent,
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32 puromycin-resistant colonies were obtained in a 1NMPP1-sensitive manner (Table 3).
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34 To confirm that their survival was dependent on xCdk1-AS/AF, three of these clones
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36 (AS/AF4, 6 and 7) were expanded and treated with 1NMPP1 (Figure 5). In two clones
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38 (AS/AF6 and 7), proliferation was impaired and polyploid cells accumulated. The
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40 third clone (AS/AF4) was incompletely sensitive to 1NMPP1, however, suggestive of
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42 clonal impurity, possibly due to upregulation of residual hCdk1 activity in a minority
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44 of cells (40). The low frequency of complementation by xCdk1-AS/AF (Table 3)
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46 relative to xCdk1-AS (Table 1) suggests that, even when driving xCdk1-AS/AF
47
48 expression with the *CDK1* promoter, only those clones expressing the smallest
49
50 amounts of xCdk1-AS/AF are viable. Consistent with this, immunoblots showed that
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52 xCdk1 was less abundant in xCdk1-AS/AF-rescued clones than in AS5 (Figure 5C).
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3 In previous work, HT2-19 cells rescued by exogenous hCdk1AF appeared to
4 depend on residual endogenous hCdk1 for viability (39). We were therefore interested
5 to know whether the same was true in cells rescued by xCdk1-AS/AF. To test this we
6 treated AS/AF7 cells with RO3306, in order to inhibit residual endogenous hCdk1
7 activity, and found that proliferation was unaffected, even though the cells remained
8 sensitive to 1NMPP1 (Figure 6A). Moreover, when a late passage of clone AS/AF4,
9 which had become largely insensitive to 1NMPP1, was challenged with RO3306,
10 proliferation slowed temporarily and the emerging population showed renewed
11 sensitivity to 1NMPP1 (Figure 6B). Thus RO3306 can be used to enrich for xCdk1-
12 rescued cells at the expense of any xCdk1-independent cells. These results show that
13 proliferation of mammalian cells does not depend on the ability of Cdk1 to undergo
14 inhibitory phosphorylation of T14 and Y15.
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Discussion

Cell cycle control by Cdk1 is fundamental to eukaryotic biology, and an attractive target for the control of malignant disease (1-5), but it remains incompletely understood. In this study we have established a chemical genetic system for specifically inhibiting Cdk1 activity in mammalian cells, and have used it to study the effects of quantitative variations in Cdk1 activity and the importance of inhibitory Cdk1 phosphorylation for cell proliferation.

Our system was based on human HT2-19 cells that undergo endoreduplication and apoptosis when endogenous Cdk1 is depleted. These cells displayed a similar phenotype when rescued with a *Xenopus*-derived, analogue-sensitive Cdk1 (xCdk1-AS) and treated with the ATP analogue 1NMPP1. Thus, while enzyme inhibition and depletion can have different biochemical and phenotypic effects (41,42), this does not appear to be the case for Cdk1. Even when xCdk1-AS was greatly overexpressed (e.g. in clones AS1 and AS4), the same 1NMPP1-induced phenotype was observed, and was dominant over endogenous hCdk1. The same clones grew normally in the absence of 1NMPP1, albeit somewhat slower than parental cells. These results suggest that excess xCdk1 out-competes endogenous Cdk1 for binding to its normal cyclin partners, and has minimal non-physiological interactions with other host proteins.

Based on the quantitative model of Cdk1 action, we expected that partial inhibition of Cdk1 might prevent its ability to promote mitosis but not to suppress endoreduplication. Over a range of 1NMPP1 concentrations, however, none was found to prevent mitosis without also inducing endoreduplication. In principle this could be explained if HT2-19 cells endoreduplicate only after entering but failing to

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3 complete mitosis, as appears to happen in some cells, including chicken B cells with
4 inhibited Cdk1(21), and spontaneously endoreduplicating megakaryocytes and
5 trophoblasts (43). Other cells, however, including primary mouse fibroblasts (16,44)
6 and HT2-19 cells (45), endoreduplicate from G2, without entering mitosis. In the
7 quantitative model, therefore, the threshold of Cdk1 activity required to suppress
8 endoreduplication in HT2-19 cells is expected to be lower than that for promoting
9 mitosis, so that an intermediate level of Cdk1 activity should exist that prevents
10 endoreduplication without promoting mitosis, resulting in G2 arrest. The failure to
11 detect robust G2 arrest at any 1NMPP1 concentration suggests that, at least in HT2-19
12 cells, preventing endoreduplication without promoting mitosis is achieved by
13 qualitative rather than quantitative differences in Cdk1 activity. We cannot rule out,
14 however, that quantitative control is most typical, and that HT2-19 cells are a special
15 case where the thresholds for preventing endoreduplication and promoting mitosis
16 happen to coincide. It will in any case be important to define the mechanism used by
17 HT2-19 cells to prevent endoreduplication prior to mitosis, and to determine whether
18 it applies in other cell types. If the inability of Cdk1 inhibitors to prevent mitosis
19 without also inducing endoreduplication is seen in various cell types, this will need to
20 be taken into account in the therapeutic use of Cdk1 inhibitors so as to avoid the risk
21 of inducing genome instability in normal or tumour cells.
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45 An attractive model to explain the observed link between inhibiting mitosis
46 and inducing endoreduplication can be found by considering the subcellular
47 localisation of the only essential Cyclin partners for Cdk1, CyclinA2 and CyclinB1
48 (13). CyclinA2 is the most likely Cdk1 partner involved in preventing
49 endoreduplication because its nuclear location during G2 (46) allows access to the
50 replication origin licensing factors implicated in mediating the suppression of
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3 endoreduplication by Cdk1 (47). In contrast, Cyclin B1 is cytoplasmic during G2
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5 (46), and so prevented, until nuclear membrane breakdown, from acting on the
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7 nuclear Cdk1 targets implicated in promoting mitosis. Our results may therefore be
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9 explained by the co-inhibition of CyclinA2/Cdk1 and CyclinB1/Cdk1 by 1NMPP1,
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11 making it impossible to prevent mitosis without also permitting endoreduplication.
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13 This model could be qualitative or quantitative in that CyclinA2/Cdk1 and
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15 CyclinB1/Cdk1 may have different substrate specificities or similar specificities but
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17 different levels of activity. Another possibility is that there are indeed two distinct
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19 thresholds, as predicted in the quantitative model, but that cells maintain Cdk1
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21 activity at a level between these thresholds only briefly before a mechanism is
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23 activated allowing cyclin degradation without the normal requirement for reaching
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25 anaphase. Further chemical genetic studies involving Cdk1-Cyclin fusion proteins and
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27 more detailed analyses of cell cycle kinetics are required to explore these possibilities.
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32 The chemical genetic approach used in this study relied on the unexpected
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34 observation that the F80G mutation confers 1NMPP1-sensitivity more effectively in
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36 xCdk1 than in hCdk1. We also discovered a further unexpected benefit in using
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38 xCdk1, namely that rescued cells are resistant to 0.5 μ M RO3306. This may facilitate
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40 chemical genetic analyses of Cdk1 in other RO3306-sensitive host cells by avoiding
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42 the need to modify host cell *CDK1* alleles. The basis of RO3306-resistance in xCdk1-
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44 rescued cells requires further investigation, however, given that xCdk1
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46 immunoprecipitated from *Xenopus* egg extracts is inhibited by 0.5 μ M RO3306 (48).
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48 This apparent discrepancy could be explained if xCdk1 is more RO3306-resistant
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50 when bound to human cyclins than *Xenopus* cyclins, although a structural basis for
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52 this is hard to envisage. Alternatively, differences (e.g. in ATP concentrations)
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54 between *in vitro* assay conditions and the cellular environment may be responsible.
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3 Here we exploited the RO3306-resistance of xCdk1-rescued cells to clarify the
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5 role of inhibitory phosphorylation of Cdk1. Although theoretical models predict that
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7 inhibitory phosphorylation of Cdk1 need not be essential for establishing sustained
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9 cell cycle oscillations (49-51), most experimental evidence has been to the contrary
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11 (29-37). Our previous observation that Cdk1-depleted HT2-19 cells could be rescued
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13 by a hCdk1AF provided the first experimental evidence in support of a non-essential
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15 role for inhibitory phosphorylation (38). Further analysis, nevertheless, suggested that
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17 rescue by hCdk1AF was impaired when the small amount of residual endogenous
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19 Cdk1 was depleted by RNA interference (39). In the present study, however, rescue
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21 of Cdk1-depleted HT2-19 cells by xCdk1-AS/AF was not only sensitive to INMPP1
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23 but also resistant to RO3306, indicating that xCdk1-AS/AF is able to support cell
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25 proliferation without the assistance of any endogenous Cdk1. The apparent difference
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27 in the abilities of hCdk1AF and xCdk1AS/AF to complement Cdk1-depleted cells
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29 may be genuine. Thus full complementation may only be possible within a narrow
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31 window of Cdk1AF expression, and this may be more difficult to achieve with
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33 hCdk1AF than with xCdk1-AS/AF. Alternatively, although the siRNA used to deplete
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35 residual Cdk1 did not affect hCdk1/AF expression (39), it may have impaired
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37 proliferation via other off-target effects.
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43 Our results suggest that the basic cell cycle, in mammalian cells as in fission
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45 yeast, can support cell proliferation without the need for inhibitory phosphorylation of
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47 Cdk1. This conclusion contrasts with previous reports showing that transient
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49 expression of Cdk1AF causes aberrant mitoses (33,37) suggesting that inhibitory
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51 phosphorylation is indispensable. Transient expression, however, allows little control
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53 over the level of Cdk1AF, which is clearly a key variable in its influence on the cell
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55 cycle. Furthermore, transient expression only allows the effects of gene expression to
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3 be observed for a limited number of cycles. In contrast, our analysis depends on the
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5 stable expression of Cdk1AF at levels that are not only compatible with, but also
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7 necessary for, cell viability. The fact that inhibitory phosphorylation is so well
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9 conserved presumably reflects its importance in both mediating G2-arrest as a
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11 response to DNA damage (52,53) and refining the efficiency of the fundamental Cdk-
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13 based cell cycle oscillator (36). These roles are almost certainly required to ensure
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15 genome stability during extensive cell proliferation, and it will be interesting to assess
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17 genome stability in clones such as AS/AF7. Given that the first Cdk-based cell cycles
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19 to evolve presumably lacked the ability to undergo inhibitory phosphorylation, it is
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21 less surprising that such simplified cycles can be effective than that they can still be
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23 effective in human cells. The ability of cell proliferation to persist after ablating the
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25 mechanism responsible for the late G2 surge in Cdk1 activity suggests again that the
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27 potential of therapies based on inhibiting only Cdk1 may be limited.
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Materials and Methods

Construction of Cdk1 expression plasmids.

The key plasmids used in this study are summarised in Table 4.

Human Cdk1 (hCdk1) expression plasmids were as follows. To express hCdk1 and hCdk1-AS a derivative of pBSgptCDK1-WT-HA (39) lacking the haemagglutinin tag was made (pBSgpt/CDC) and oligonucleotide-mediated mutagenesis (QuikChange, Agilent) was used to introduce the F80G (TTT>GGT) mutation, generating pBSgpt/CDC2as. The 6.3 kb BamH1 fragments from pBSgpt/CDC and pBSgpt/CDCas were then cloned into the BamH1 site of pBL-Puro (54) to generate pPuroCDC and pPuroCDC2as, respectively. To express hCdk1 with an F80A mutation (hCdk1-AS'), a PCR mutagenesis protocol was used to introduce the F80A mutation (TTT>GCT) into pPuroCDC, generating pPuroCDC2as'.

Xenopus laevis Cdk1 (xCdk1) expression plasmids were as follows. The previously described (21) constructs for expressing xCdk1-WT and xCdk1-AS were used initially. These (here named pXICdkWT-mycEGFP and pXICdkAS-mycEGFP) contained *Xenopus laevis* Cdk1myc cloned into pIRES2-EFGP (Clontech) with or without the F80G mutation (TTT>GGT). To express xCdk1-AS/AF, QuikChange mutagenesis was used to introduce the AF mutation (T14A, Y15F; ACATAT>GCATTT) into pXICdkAS-mycEGFP, generating pXICdk1AS/AF-mycEGFP. To allow co-selection for Puromycin resistance, these three plasmids were cotransfected with pBL-Puro (54). Alternatively they were modified to carry a puromycin resistance cassette by linearizing at their unique AflIII site, end-filling and ligating to a 1.4 kb EcoRI/XhoI end-filled fragment from pBL-Puro, generating pXICdkWT-mycEGFP-puro, pXICdkAS-mycIRESeGFP-puro and

1
2
3 pXICdkAS/AFmycIRESeGFP-puro. Finally, to place xCdk1-AS/AF expression under
4
5 the control of the human Cdk1 promoter, an end-filled 7kb AseI/EcoR1 fragment was
6
7 isolated from pXICdkAS/AFmycIRESeGFP-puro and ligated to an end-filled 3.6 kb
8
9 BamHI/NotI fragment from pPuroCDC, to generate pXICdkAS/AFmycIRESeGFP-
10
11 puro-HP.
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13 14 15 16 *Cell growth and transfection of HT2-19 cells and derivatives*

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18 Growth and transfection of cells was as previously described (40). IPTG (2mM final
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20 concentration) was added to the medium for routine growth of HT2-19, but removed,
21
22 unless indicated otherwise, during selection and maintenance of transfectants. For
23
24 transfections, up to 10^7 cells were electroporated with 10 μ g FspI-linearised plasmid
25
26 DNA and distributed in 10 cm diameter petri dishes at $\sim 10^6$ cells per dish. After 24-48
27
28 hours selection was initiated. Where required, puromycin (InvivoGen) was added to a
29
30 final concentration of 0.4 μ g/ml. Stock solutions of 1NMPP1 (Toronto Research
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32 Chemicals) and RO3306 (Calbiochem) were dissolved in dimethyl sulphoxide before
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34 diluting in medium to the indicated final concentrations.
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41 42 *Generation of 1NMPP1-sensitive clones*

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44 HT2-19 cells were transfected with 10 μ g (unless stated otherwise) of the following
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46 plasmids. Table 1: pXICdkWT-mycEGFP-puro or pXICdkAS-mycIRESeGFP-puro
47
48 (both linearised with FspI). Table 2: pXICdkWT-mycEGFP or pXICdkAS-
49
50 mycIRESeGFP (each cut with PvuII and cotransfected with 1 μ g pBL-Puro). Table 3:
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52 SpeI-linearised pXICdkAS-mycIRESeGFP-puro or BstB1-cut
53
54 pXICdkAS/AFmycIRESeGFP-puro-HP. Table S1: SpeI-linearised pPuroCDC or
55
56 pPuroCDCas. Table S2: SpeI-linearised (5 μ g) pPuroCDC or pPuroCDCas'.

Growth Curves

Known numbers of cells, e.g. 25000, 12500 and 5000, cells were plated into three wells (2.5 cm diameter) of a 12-well plate, harvested 2, 4 or 7 days later, respectively, and counted in a haemocytometer to determine the number of doublings. Error bars show the average and standard deviations for at least three independent experiments.

Flow Cytometry

For DNA content analysis nuclei stained with propidium iodide (PI) were prepared as previously described (40). Samples were incubated overnight at 4°C and then analysed on a FACS Calibur (BD Biosciences) using the 488nm laser. For GFP expression analysis, cells were trypsinised and resuspended in cold phosphate buffered saline solution and analysed immediately.

Western Blotting

Cells were lysed in RIPA buffer (0.75M NaCl, 5%v/v NP40, 2.5% v/v DOC, 0.5% w/v SDS and 0.25M Tris, pH8) containing protease inhibitors (Complete; Roche). Samples (40 µg total protein each) were separated on SDS-polyacrylamide gels and transferred to Hybond ECL membranes (Amersham). Membranes were probed overnight with the following antibodies diluted in 5% milk: mouse anti-Cdc2p34 (Santa Cruz biotechnology; 1:1000 dilution), rabbit anti-c-myc (Abcam, 1:10,000 dilution), mouse anti-GFP (Roche, 1:1000 dilution) or rabbit anti-β-tubulin (Cell Signalling, 1:1000 dilution). After the addition of the appropriate species-specific horseradish peroxidase-conjugated secondary antibody and further washes, proteins

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2
3 were visualised using ECL reagents (Thermo Scientific) following manufacturers'
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5 instructions.
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12
13
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15
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17
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19
20 Humphrey for their comments on the manuscript.
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28 **Conflict of Interest**

29
30 The authors declare that they have no conflict of interest.
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Legends to Figures

Figure 1. *Xenopus* Cdk1-AS rescues HT2-19 cells and confers 1NMPP1

sensitivity. A. Growth curves are shown for one xCdk1-WT-rescued clone (WT1), three xCdk1-AS-rescued clones (AS1, AS4, AS5) and parental HT2-19 cells in the presence or absence of 10 μ M 1NMPP1 or 2 mM IPTG, as indicated. **B.** The xCdk1-rescued clones in A were analysed for DNA content by flow cytometry of propidium iodide-stained nuclei after culturing in 10 μ M 1NMPP1 for the indicated times. Tetraploid (8N) peaks are marked (\diamond).

Figure 2. Analyses of protein expression in xCdk1-rescued clones. A. The same clones as in Figure 1 were grown in the presence (-) or absence (+) of IPTG for 7 days before immunoblotting lysates with a Cdk1 antibody that detects hCdk1 and xCdk1. The same blot was re-probed to detect β -tubulin (β -tub). **B.** The lysates described in A (-IPTG samples) were immunoblotted and probed with antibodies to c-myc, GFP or β -tubulin, as indicated. **C.** Flow cytometric analyses of GFP in cells from the same cultures analysed in B.

Figure 3. Cells expressing xCdk1 are resistant to the Cdk1 inhibitor RO3306. A. HT2-19 cells (left) or AS5 cells (right) were grown for 72h in medium containing 0, 0.5 or 1.0 μ M RO3306, as indicated, and analysed for DNA content. 2N (*), 4N (=), 8N (\diamond) and 16N (\square) peaks are marked. **B.** Cell proliferation was monitored in cultures of WT1 (top) or AS5 (bottom) supplemented with 0.5 μ M RO3306, with or without 10 μ M 1NMPP1 as indicated.

Figure 4. Endoreduplication and proliferation of xCdk1-AS-rescued clones at different 1NMPP1 concentrations. A. Clones WT1, AS1, AS4 and AS5 were

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3 treated with the indicated concentrations of 1NMPP1 and analysed for DNA content
4 by flow cytometry at the indicated times after 1NMPP1 addition. Shown are 4N/2N
5 (top) and 8N/2N (bottom) ratios of DNA content. **B.** Growth curves are shown for
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7 WT1, AS1, AS4 and AS5 in medium with no 1NMPP1 or with concentrations of
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9 1NMPP1 between 0 and 10 μ M.
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15 **Figure 5. Clones rescued with xCdk1-AS/AF are sensitive to 1NMPP1. A.** Growth
16 curves are shown for three xCdk1-AS/AF-rescued clones (AS/AF4, AS/AF6, AS/AF7)
17 grown in medium with concentrations of 1NMPP1 between 0 and 10 μ M. **B.** DNA
18 content analysis of the indicated clones grown in the presence (+) or absence (-) of
19 10 μ M 1NMPP1 for 7 days. Peaks of fluorescence are labelled as in Figure 3. **C.**
20 Immunoblot detection of xCdk1-AS/AF and endogenous hCdk1 in the indicated
21 clones. All lysates except that in lane 1 were from cultures grown without IPTG.
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32 **Figure 6. AS/AF clones proliferate in RO3306 and maintain sensitivity to**
33 **1NMPP1. A.** (Top) Growth of AS/AF7 cells in medium with or without 10 μ M
34 1NMPP1 (NM) or 0.5 μ M RO3306 (RO), as indicated. (Bottom) DNA content
35 analyses of cells harvested at day 7. **B.** Clone AS/AF4 was passaged for 2 weeks in
36 the absence of RO3306. This late passage culture (AS/AF4(lp)) was then split,
37 passaging one culture with and one without RO3306 (0.5 μ M) for a further 12 days,
38 the last 7 days being in the presence or absence of 10 μ M 1NMPP1 and monitored
39 for cell numbers (top). DNA content analyses of cells harvested at day 7 are shown
40 (bottom).
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Table 1. Complementation of HT2-19 by xCdk1-AS

^a No. Cells	Selection reagent			^b Colonies	
	Puromycin	IPTG	1NMPP1	WT	AS
10 ⁶	+	+	-	37 (100%)	78 (100%)
10 ⁶	+	-	-	32 (86%)	54 (69%)
10 ⁶	+	-	+	24 (64%)	0 (0%)

^a number of HT2-19 cells electroporated

^b colony numbers resulting from transfection with expression plasmids for xCdk1-WT (WT) or xCdk1-AS (AS).

Table 2. Preliminary analysis of HT2-19 clones rescued by xCdk1-WT or xCdk1-AS

^a Clone	^b 1NMPP1-sensitivity			^c GFP	^a Clone	^b 1NMPP1-sensitivity		
	- IPTG	+ IPTG				- IPTG	+ IPTG	
WT1	-	-	++		AS1	+	+	++
WT2	-	-	-		AS2	+	+	++
WT3	-	-	-		AS3	+	+	NT
WT4	-	-	+		AS4	+	+	+
WT5	-	-	-		AS5	+	-	-
WT6	-	-	+		AS6	+	+	++
WT7	-	-	-		AS7	+	+	+
					AS8	+	+	+
					AS9	+	+	++
					AS10	+	+	+
					AS11	+	+	+
					AS12	+	+	+
					AS13	+	+	+

^a WT and AS colonies selected in puromycin but no IPTG after transfection with xCdk1-WT or xCdk1-AS expression constructs, respectively, were analysed.

^b Proliferation judged by phase-contrast microscopy was unaffected (+) or impaired (-) by addition of 10 μ M 1NMPP1 in the presence or absence of IPTG.

^c GFP expression judged by fluorescence microscopy was strong (++) , detectable (+) or undetectable (-). NT = not tested.

Table 3. Complementation of HT2-19 by xCdk1-AS/AF

^a No. Cells	Selection reagent			^b Colonies	
	Puromycin	IPTG	1NMPP1	AS	AS/AF
10 ⁶	+	+	-	140 (100%)	123 (100%)
3x10 ⁶	+	-	-	239 (57%)	12 (3%)
3x10 ⁶	+	-	+	0 (0%)	0 (0%)

^a number of HT2-19 cells electroporated

^b colony numbers resulting from transfection with expression plasmids for xCdk1-AS (AS) or xCdk1-AS/AF (AS/AF).

Table 4. Summary of plasmids

Plasmid Name	Cdk1 encoded	^a Pro	^b Mark	^c Ref
pBL-Puro	-	-	Puro	1
pBSgptCDK1-WT-HA	hCdk1-WT	hCdk1	Gpt	2
pBSgpt/CDC	hCdk1-WT	hCdk1	Gpt	
pBSgpt/CDCas	hCdk1-AS	hCdk1	Gpt	
pPuroCDC	hCdk1-WT	hCdk1	Puro	
pPuroCDCas	hCdk1-AS	hCdk1	Puro	
pPuroCDCas'	hCdk1-AS'	hCdk1	Puro	
pXICdkWT-mycEGFP	xCdk1-WT	CMV	Neo	3
pXICdkAS-mycEGFP	xCdk1-AS	CMV	Neo	3
pXICdkAS/AF-mycEGFP	xCdk1-AS/AF	CMV	Neo	
pXICdkWT-mycEGFP-puro	xCdk1-WT	CMV	Neo, Puro	
pXICdkAS-mycEGFP-puro	xCdk1-AS	CMV	Neo, Puro	
pXICdkAS/AFmycEGFP-puro	xCdk1-AS/AF	CMV	Neo, Puro	
pXICdkAS/AFmycEGFP-puro-HP	xCdk1-AS/AF	hCdk1	Neo, Puro	

^a Promoters driving Cdk1 expression: CMV, cytomegalovirus immediate early promoter; hCdk1, promoter region from human Cdk1 gene.

^b Markers encoding proteins (Gpt, guanosine-hypoxanthine phosphoribosyltransferase; Puro, puromycin N-acetyl-transferase; Neo, aminoglycoside 3'-phosphotransferase) conferring resistance to mycophenolic acid, puromycin and neomycin, respectively.

^c 1) (54). 2) (39) 3) (21).

Abbreviations

1NMPP1: 4-Amino-1-tert-butyl-3-(1'-naphthylmethyl)pyrazolo[3,4-d]pyrimidine. AS: analogue-sensitive. Cdk: Cyclin Dependent Kinase. siRNA: small interfering ribonucleic acid. WT: wild-type.

For Peer Review

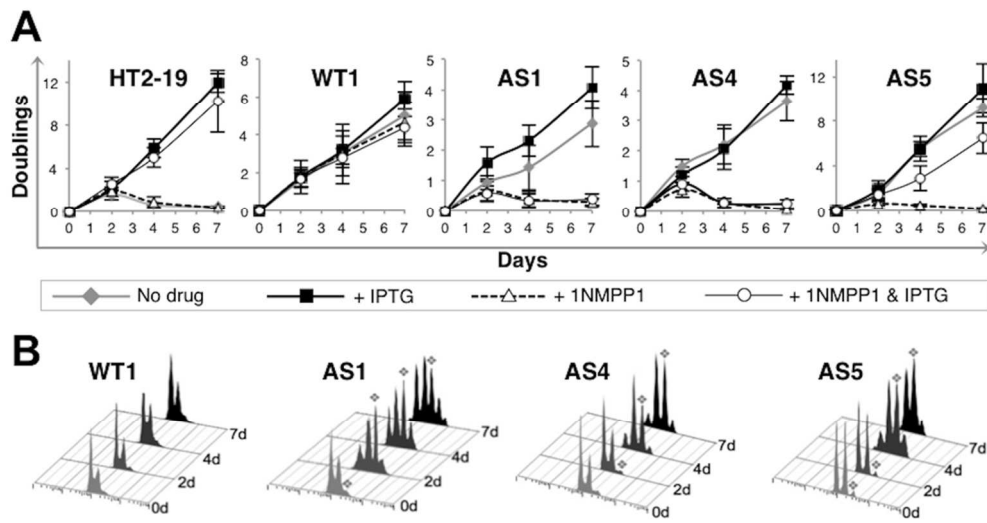


Figure 1. *Xenopus* Cdk1-AS rescues HT2-19 cells and confers 1NMPP1 sensitivity.
95x50mm (300 x 300 DPI)

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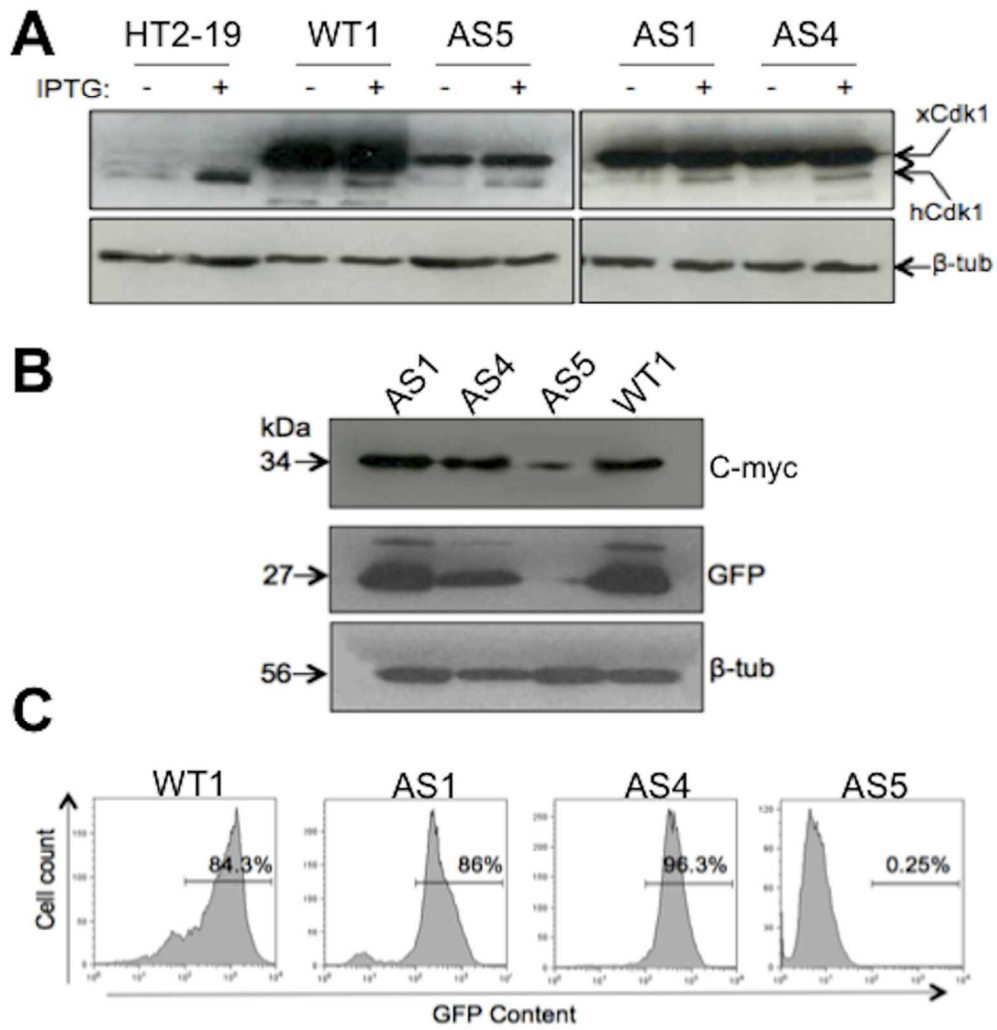


Figure 2. Analyses of protein expression in xCdk1-rescued clones.
89x93mm (300 x 300 DPI)

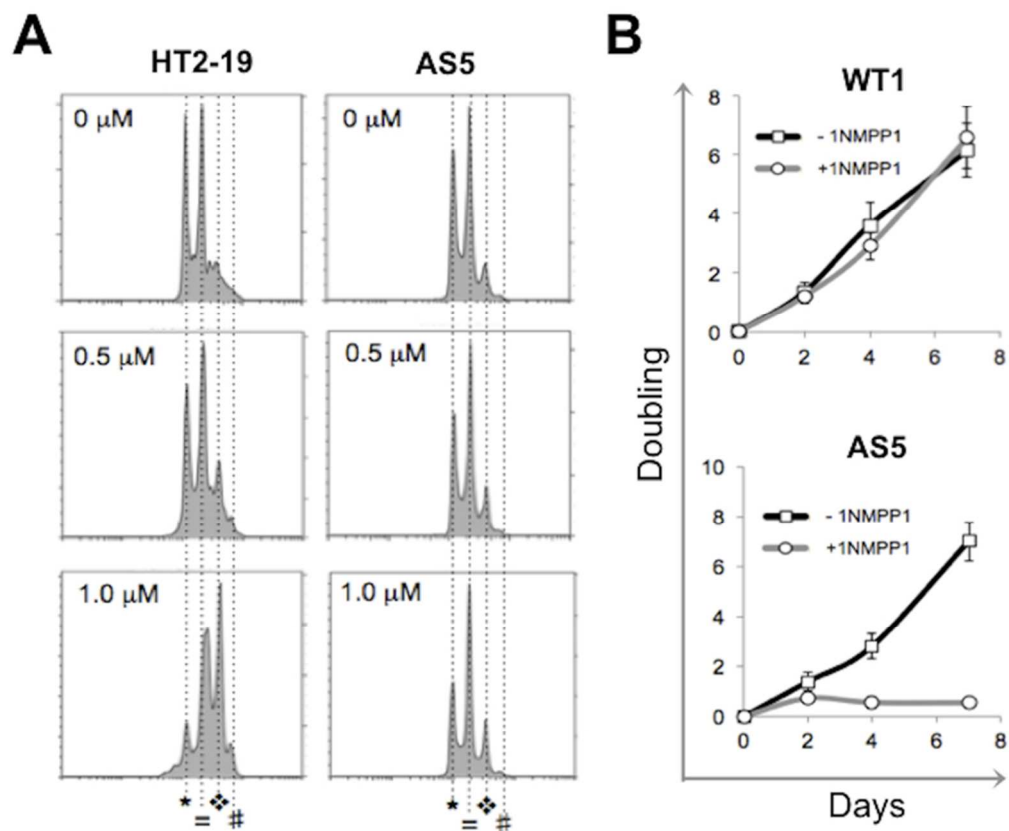


Figure 3. Cells expressing xCdk1 are resistant to the Cdk1 inhibitor RO3306.
71x59mm (300 x 300 DPI)

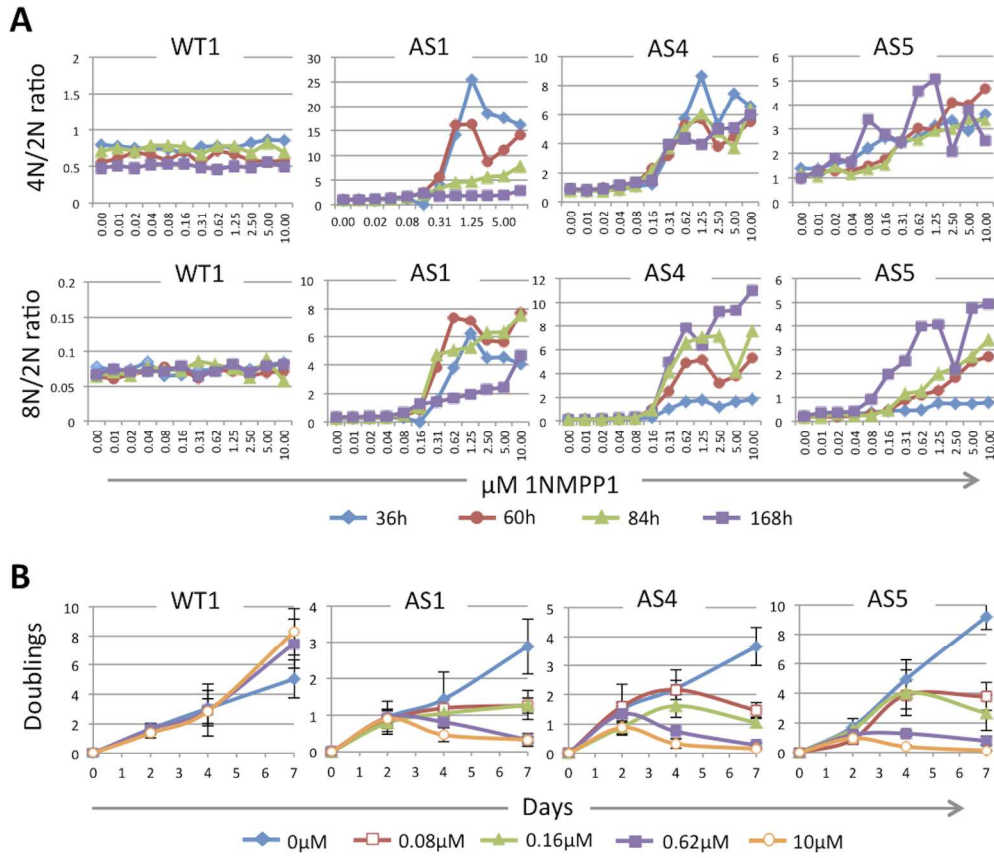


Figure 4. Endoreduplication and proliferation of xCdk1-AS-rescued clones at different 1NMPP1 concentrations.
180x154mm (300 x 300 DPI)

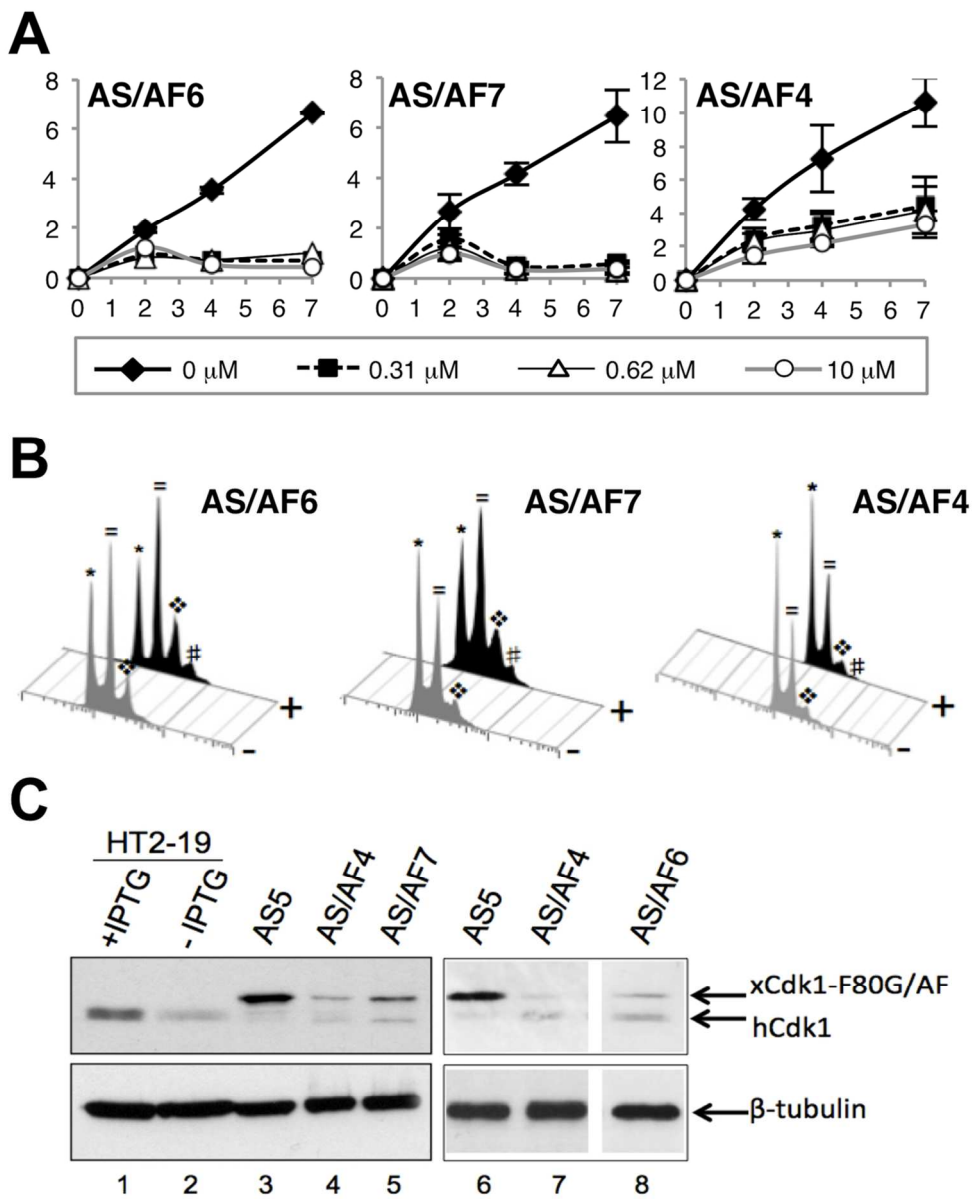
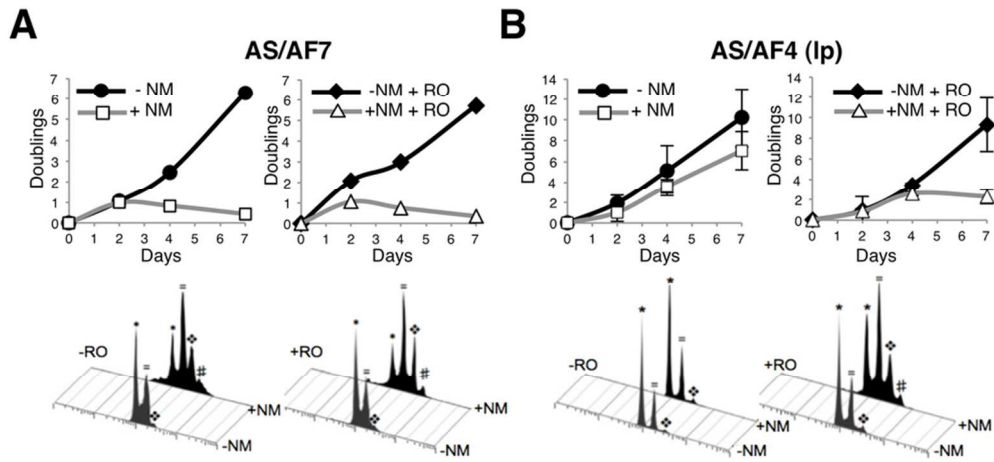


Figure 5. Clones rescued with xCdk1-AS/AF are sensitive to 1NMPP1.
105x130mm (300 x 300 DPI)

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84x39mm (300 x 300 DPI)

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